REMARKS

Interview request

Applicants respectfully request a telephonic interview after the Examiner has reviewed the instant response and amendment. Applicants request the Examiner call Applicants' representative at 858 720 5133.

Status of the Claims

Pending claims

Claims 1 to 13 and 16 to 46 are pending.

Claims added and amended in the instant amendment

Claims 2, 10 to 12, 16, 21 to 23, 35 to 43, and 46 have been amended and claims 47 to 76 are added. Claim 44 has been canceled without prejudice or disclaimer. Thus, after entry of the instant amendment, claims 1 to 13, 16 to 43 and 45 to 76 will be pending.

Outstanding Rejections

Claims 21 to 23 and 34 remain rejected and claims 35 to 44, and 46 stand newly rejected under 35 U.S.C. §112, second paragraph. Claims 1 to 13 and 16 to 46 stand rejected under 35 U.S.C. §112, first paragraph. Claims 1 to 13, 16-27, 29 to 32 and 34 and new claims 35-46 stand provisionally rejected under the judicially created doctrine of obviousness-type double patenting.

Applicants respectfully traverse all outstanding objections to the specification and rejection of the claims. Reconsideration of the pending claims is respectfully requested.

Support for the Claim Amendments

The specification sets forth an extensive description of the invention in the new and amended claims. For example, claims directed to compositions comprising nucleic acids or methods comprising use of nucleic acids having at least about 50% sequence identity, which would include, for example, at least 70%, 80% or 90% sequence identity, to SEQ ID NO:2, can be found, inter alia, on page 20, lines 12 to 20. Support for claims directed to compositions comprising various types of vectors or methods comprising use of various types of vectors can be found, inter alia, on page 37, lines 7 to 14; page 39, lines 3 to 14; page 41, first and second paragraphs. Support

for claims directed to compositions comprising various types of cells or methods comprising use of various types of cells can be found, inter alia, on page 38, fourth paragraph; page 39, last paragraph; the paragraph spanning pages 41 and 42. Support for claims directed to compositions comprising various types of promoters or methods comprising use of various types of promoters can be found, inter alia, on page 39, lines 15 to 21. Support for claims directed to compositions comprising various types of plant cells or methods comprising use of various plant cells can be found, inter alia, in the specification from pages 44 to 49. Accordingly, no new matter has been added by way of these amendments and support for each amendment can be found in the original claims as filed and throughout the specification.

Objections to the Specification

The Examiner requests clarification regarding the presence of a His tag in SEQ ID NO: 2. The Examiner is correct in her understanding that SEQ ID NO: 2 contains an open reading frame encoding a protein of 432 amino acids - amino acid 432 being leucine. The His tag comprises 6 hisitidine residues preceded by an Arg-Ser linker sequence. Another example of a linker sequence is shown in Wyss, M., *et al.*, Applied and Environmental Microbiology (1999) 65(2): 359-366, on page 4, second full paragraph, where it is described how "[t]he *appA* gene was transferred into the pQE60 expression vector (Qiagen) containing a C-terminal 6xHis tag and a short linker sequence (Gly-Ser-Arg-Ser-His-His-His-His-His-His)." Thus, the linker sequence is not a part of the coding sequence but merely links the amino acid sequence encoding the protein to the amino acid sequence that is the 6xHis tag. An online version of this article is provided for the convenience of the Examiner.

Objections to the Claims

Claim 16 is objected to for the presence of the second occurrence of the word "and". Accordingly, the second "and" in the claim has been removed.

Claim Rejections - 35 U.S.C. §112, Second Paragraph

Claims 21 to 23 and 34 remain rejected and claims 35 to 44, and 46 stand newly rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Patent Office alleged that claim 21 is indefinite in the recitation of "wherein the nucleic acid further comprises a vector sequence" for the reasons previously set forth in the Office Action mailed February 17, 2004. Applicant has amended claim 21 to read "The expression system of claim 1 or 16, wherein the nucleic acid is contained in a vector."

Claims 22-23 are alleged as being indefinite in the recitation of "wherein the vector comprises a cloning vector, an expression vector...." Claims 22 and 23 have been amended to clarify that the vector can comprise at least a portion of a nucleotide sequence taken from a cloning vector, an expression vector, a bacterial vector, a plasmid, a viral particle, a phage, chromosomal DNA, nonchromosomal DNA, synthetic DNA, a vaccinia vector, an adenovirus vector, a fowl pox virus, a pseudorabies vector, or a combination of more than one nucleotide sequence taken from the sources listed above.

The Patent Office alleged that claim 34 is indefinite in the recitation of "wherein the phytase activity comprises hydrolyzing inorganic phosphate from phytate" as it does not further limit claim 16. Applicant has amended claim 16 to recite the phrase "a polypeptide having phytase activity, wherein the phytase activity comprises hydrolyzing inorganic phosphate from phytate or the reverse reaction".

Claims 35-44 and 46 are alleged to be indefinite in the recitation of "comprising a sequence that is the complement of a sequence of ..." because it is unclear which complement is being referred to. Although Applicant respectfully traverse, only to expedite prosecution claims 35-43 and 46 have been amended to clarify which complement is being referred to. Claim 44 is canceled in the instant amendment.

Accordingly, Applicant asserts that the language of claims 21-23, 34, 35-44, and 46 is clear and meets the requirements of 35 U.S.C. § 112, second paragraph.

Issues under 35 U.S.C. §112, first paragraph

Written Description

New matter

Claims 1 to 13 and 16 to 46 stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the written description requirement and containing subject matter

not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor had possession of the claimed invention.

Regarding claims 1-13, and 16-46 (see paragraph 10, page 5, of the instant office action), the Patent Office alleged that there is inadequate support in the specification for an expression system, a cell, or a vector comprising a polynucleotide encoding amino acids 1-432 of SEQ ID NO: 2. It is alleged that there is no support in the specification for a method for making a phytase with a cell comprising a polynucleotide encoding amino acids 1-432 of SEQ ID NO: 2.

However, Applicants respectfully note that there several places in the specification in which SEQ ID NO: 2 is described as comprising the coding sequence for an enzyme and an additional sequence, *e.g.* a 6xHis tag. For example, paragraph 101 of published patent application US 2001/0055788 states that "[a]n analog, derivative, or fragment of the enzyme of FIG. 1 may be ... (d) to provide a label or a tag, such as a 6xHis tag ... . Also, paragraph 115 of the published application describes how

> "[t]he polynucleotide which encodes for the mature enzyme of FIG. 1 (*e.g.*, SEQ ID NO: 2) may include, but is not limited to: only the coding sequence for the mature enzyme; the coding sequence for the mature enzyme and additional coding sequence such as a leader sequence or a proprotein sequence; the coding sequence for the mature enzyme (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature enzyme."

In paragraphs 121-125 of the published application, it is discussed how "[t]he coding sequences for the phytase enzymes of the present invention were identified by ... and recovering ... DNA encoding phytase activity. It is also described how the recovered DNA is then ligated and transformed "into, and express[ed] in M15pREP4 host cells (Qiagen) yield[ing a] C-term 6X-His tagged protein."

Accordingly, Applicants respectfully aver that claims directed to expression systems, cells, or vectors comprising a polynucleotide encoding amino acids 1-432 of SEQ ID NO: 2, and methods for making a phytase with a cell comprising a polynucleotide encoding amino acids 1-432 of SEQ ID NO: 2, are supported in the specification and no new matter has been added by amendment.

Possession of the claimed invention

Claims 2, 10 to 13, 16 to 17, and 20 to 34 remain rejected and new claims 35-44 and 46 are newly rejected as allegedly containing subject matter not described in the specification in such as way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

Applicants respectfully aver that the claimed invention is sufficiently described in the specification such that one of ordinary skill in the art would be able to ascertain the scope of the claims with reasonable clarity and recognize that Applicants' were in possession of the claimed invention at the time of filing. Applicants respectfully submit that the present amendment addresses all of these issues.

The Federal Circuit has applied the written description requirement of the first paragraph of § 112 to inventions in the field of biotechnology. See University of California v. Eli Lilly and Co., 119 F.3d 1559, 1568, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997). The court explained that

> In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus. . . [H]owever, a generic statement such as "vertebrate insulin cDNA" or "mammalian insulin cDNA," without more, is not an adequate written description of the genus because it does not distinguish the claimed genus from others, except by function. It does not specifically define any of the genes that fall within its definition. It does not define any structural features commonly possessed by members of the genus that distinguish them from others. One skilled in the art therefore cannot, as one can do with a fully described genus, visualize or recognize the identity of the members of the genus. A definition by function, as we have previously indicated, does not suffice to define the genus because it is only an indication of what the gene does, rather than what it is.

Id. at 1568, 43 USPQ2d at 1406.

The Lilly court also stated that "[a] written description of an invention involving a chemical genus, like a description of a chemical species, 'requires a precise definition, such as by structure, formula, [or] chemical name,' of the claimed subject matter sufficient to distinguish it from other materials." Id. at 1567, 43 USPQ2d at 1405. Finally, the court addressed the manner by

which a genus of cDNAs might be described. "A description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus." Id. at 1568, 43 USPQ2d at 1406.

The Federal Circuit has also addressed the written description requirement in the context of DNA-related inventions. See Enzo Biochem. Inc. v. Gen-Probe Inc., 296 F.3d 1316, 63 USPQ2d 1609 (Fed. Cir. 2002). The Enzo court adopted the standard that "the written description requirement can be met by 'showing that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics . . . *i.e.*, complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.'" [Emphasis added] Id.. at 1324, 63 USPQ2d at 1613.

The court in Enzo adopted its standard from the USPTO's Written Description Examination Guidelines. See 296 F.3d at 1324, 63 USPQ2d at 1613 (citing the Guidelines). The Guidelines apply to proteins as well as DNAs.

Finally, it is well-settled that the written description requirement of 35 U.S.C. § 112, first paragraph, can be satisfied without express or explicit disclosure of a later claimed invention. See, *e.g.*, In re Herschler, 591 F.2d 693, 700, 200 USPQ 711,717 (CCPA 1979): "The claimed subject matter need not be described in haec verba to satisfy the description requirement. It is not necessary that the application describe the claim limitations exactly, but only so clearly that one having ordinary skill in the pertinent art would recognize from the disclosure that appellants invented processes including those limitations." (citations omitted). See also Purdue Pharma L.P. v. Faulding, Inc., 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Gir. 2000) ("In order to satisfy the written description requirement, the disclosure as originally filed does not have to provide in haec verba support for the claimed subject matter at issue.").

Applicants respectfully submit that the claimed invention is sufficiently described in the specification such that one of ordinary skill in the art would be able to ascertain the scope of the claims with reasonable clarity and recognize that Applicants' were in possession of the claimed

invention at the time of filing. As noted in paragraph 12, page 6, of the instant Office Action, in previous responses Applicants have fully argued this rejection, and incorporate those remarks herein. In brief, all nucleic acids of the claimed invention are described by structure (the exemplary SEQ ID NO:2, encoded e.g., by SEQ ID NO:1), a physico-chemical property (encoding polypeptides having specific conservative amino acid variations to SEQ ID NO:2 based on interchange of aliphatic residues, hydroxyl-comprising residues, acidic residues, amide residues, basic residues or aromatic residues) and function (phytase activity). Applicants respectfully submit that describing a genus of polynucleotides in terms of physico-chemical properties (e.g., encoding polypeptides having specific conservative amino acid substitutions) and function (e.g., encoding phytases) satisfies the written description requirement of section 112, first paragraph. The USPTO guidelines recognize that a genus of nucleic acids encoding polypeptides described by structure (e.g., the exemplary SEQ ID NO:2), a physico-chemical property (e.g., specific conservative amino acid substitutions based on interchange of aliphatic residues, hydroxyl-comprising residues, acidic residues, amide residues, basic residues or aromatic residues) and a defined function (e.g., phytase activity) meets the written description requirements of section 112.

The genus of nucleic acids used in the claimed compositions and methods also fully complies with the requirements for written description of a genus of nucleic acids as set forth in University of California v. Eli Lilly & Co., 43 USPQ2d 1398 (Fed. Cir. 1997). In Lilly, the Court stated that, "[a] description of a genus of cDNA may be achieved by means of a recitation of a representative number of cDNAs....*or of a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus.*" (emphasis added) Lilly, 43USPQ2d at 1406. Analogously, the specification recites structural features (specific conservative amino acid substitutions of exemplary SEQ ID NO:2) common to the members of the genus of phytases and phytase-encoding nucleic acids of the invention, which features constitute a substantial portion of the genus.

The Patent Office remains concerned that while the specification discloses one species of claimed polynucleotide genus (nucleic acids encoding SEQ ID NO: 2) the specification is allegedly completely silent in regard to which amino acid substitutions in the polypeptide of SEQ ID NO: 2 would result in a polypeptide having the same phytase activity. The Office appears

concerned that the specification does not predict which (conservative) amino acid substitutions can be made to the exemplary SEQ ID NO:2 to generate variant phytases. However, Applicants respectfully aver that predictability is not the legal standard or test for a written description rejection. It is not necessary that the specification describe the claim limitations exactly, but only so clearly that one having ordinary skill in the pertinent art would recognize from the disclosure that appellants invented the claimed subject matter. Because the phytases and nucleic acids of the invention are described by structure (the exemplary SEQ ID NO:2, encoded e.g., by SEQ ID NO:1), physico-chemical properties (e.g., encoding polypeptides having specific conservative amino acid variations to SEQ ID NO:2 based on interchange of aliphatic residues, hydroxyl-comprising residues, acidic residues, amide residues, basic residues or aromatic residues) and function (phytase activity), one having ordinary skill in the art would recognize from the disclosure that Applicants invented the claimed subject matter.

However, the instant amendment also addresses this issue. After entry of the instant amendment, the claims are directed to, inter alia, recombinant expression systems, vectors, cells (and methods using them) comprising a nucleic acid encoding a phytase that has at least one conservative amino acid substitution to SEQ ID NO:2, and the claims expressly define what is a conservative amino acid substitution within the scope of the invention, i.e., (a) a replacement, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile, (b) an interchange of the hydroxyl residues Ser and Thr, (c) an exchange of the acidic residues Asp and Glu, (d) a substitution between the amide residues Asn and Gln, (e) an exchange of the basic residues Lys and Arg, (f) a replacement among the aromatic residues Phe, Tyr.

The Patent Office also remains concerned that the specification does not teach a correlation between the structure of SEQ ID NO: 2 and phytase function. However, Applicants' respectfully aver that it was not necessary for one skilled in the art to know the correlation between structure and function of phytases to be in possession of the invention. As declared by Dr. Short in a previous office action, one of ordinary skill in the art using the teaching of the specification would have been able to make and screen for nucleic acids that encode for one or more conservative amino acid substitutions to SEQ ID NO:2, including conservative amino acid substitutions comprising (a) a replacement, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile, (b) an

interchange of the hydroxyl residues Ser and Thr, (c) an exchange of the acidic residues Asp and Glu, (d) a substitution between the amide residues Asn and Gln, (e) an exchange of the basic residues Lys and Arg, (f) a replacement among the aromatic residues Phe, Tyr, or (g) a combination thereof, and express those nucleic acids and using routine screening to determine, with predicable positive results, which of those nucleic acids encode for a polypeptide having phytase activity. Dr. Short declared that one of ordinary skill in the art using the teaching of the specification would have been able to ascertain what polypeptides, including polypeptides of having conservative amino acid substitutions, or fragments of SEQ ID NO:2 having phytase activity, were within the scope of the claims with reasonable clarity to recognize that Applicants' were in possession of the claimed invention at the time of filing.

While Applicants maintain that it was not necessary for one of ordinary skill in the art to know the correlation between the structure of SEQ ID NO: 2 and phytase function to recognize with reasonable clarity that Applicants' were in possession of the claimed invention at the time of filing, in fact, such structure-function information for phytases was available to one of ordinary skill in the art. Attached is an alignment of the phytase SEQ ID NO:2 (designated "DVSA") compared to five phytases known in the art at the time of the invention (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=2108356; http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=166519; http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=408990; http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=2108352; http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=2108354).

The alignment shows areas of conserved sequence between the phytases, with completely conserved sequence residues highlighted in yellow. The sequence alignment highlights the conserved phytase active site, RHGXRXP as described, e.g., by Wodzinski and Ullah, "Phytase", in Advantages in Applied Microbiology, vol. 42, 1996, Academic Press, Inc. (copy attached) (see also, Berka (1998) Applied and Environ. Biol. 64:4423-4427; Kerovuo (1998) Applied and Environ. Biol. 64:2079-2085) (copies attached). Accordingly, one of skill in the art at the time of the invention would have had sufficient knowledge of the structure-function relationship of phytases and phytase active sites to design variant phytase sequences.

The Patent Office also remains concerned about the size of the genus of claimed nucleic acids (including the genus of nucleic acids used in the methods of the invention). For example, in paragraph 13 on page 8 of the instant office action, the Patent Office alleges that the scope of the claimed genus of polypeptides encompasses anywhere from 0% to 99% sequence identity to the exemplary SEQ ID NO:2. Applicants respectfully traverse, and submit that the claimed genus is not so broad as to encompass anywhere near 0% sequence identity because the genus is expressly limited to only encompass polypeptides having phytase activity and an amino acid sequence as set forth in (i) SEQ ID NO:2, or, (ii) SEQ ID NO:2 from amino acid residue 1 to 432, wherein the phytase sequence of (i) or (ii) has at least one conservative amino acid substitution from SEQ ID NO:2, and the conservative amino acid substitution comprises (a) a replacement, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile, (b) an interchange of the hydroxyl residues Ser and Thr, (c) an exchange of the acidic residues Asp and Glu, (d) a substitution between the amide residues Asn and Gln, (e) an exchange of the basic residues Lys and Arg, (f) a replacement among the aromatic residues Phe, Tyr, or (g) any combination of a, b, c, d, e or f. However, to address the Office's concerns, the claimed genus (see new claims 47 to 76) is amended such that the claimed phytases are further limited such that their sequences must have at least about 50% sequence identity to SEQ ID NO:2.

Accordingly, Applicants respectfully submit that the pending claims meet the written description requirement under 35 U.S.C. §112, first paragraph. In light of the above remarks, Applicants respectfully submit that amended claims are sufficiently described in the specification to overcome the written description rejection based upon 35 U.S.C. §112, first paragraph.

Enablement

Claims 2, 10-13, 16-17, 20-34, and new claims 35-44 and 46 stand rejected under 35 U.S.C. § 112, first paragraph, enablement, .

The Patent Office states that the specification is enabling for a nucleic acid encoding the polypeptide of SEQ ID NO: 2, a vector and a host cell comprising said nucleic acid, as well as a method of recombinantly producing the polypeptide of SEQ ID NO: 2 in a cell.

However, it is alleged that the specification does not reasonably provide enablement for (1) an expression system, vector or host cell comprising a nucleic acid which encodes a phytase,

wherein said phytase has the amino acid sequence of SEQ ID NO: 2 with any number of conservative substitutions or (2) a method to produce a phytase with the host cell of (1).

In order to make a rejection, the Examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. In re Wright, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) (Examiner must provide a reasonable explanation as to why the scope of protection provided by a claim is not adequately enabled by the disclosure). A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. As stated by the court, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." In re Marzocchi, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). See also MPEP §2164.04, rev. 2, May 2004, pg 2100-189.

The Patent Office cites art to support its *prima facie* case of lack of enablement, citing Broun *et al.*(1998) Science 282:1315-1317; Witkowski *et al.*(1999) Biochemistry 38:11643-11650; and Seffernick *et al.*(2001) J. of Bacteriol. 183:2405-2410. It is alleged that this art evidences the allegation that the art teaches the unpredictability of assigning function based on structural homology and how small structural changes can lead to major changes in function. Thus, it is alleged, one of skill in the art would have to go through the burden of undue experimentation to isolate or make the nucleic acids encompassing the full scope of the invention. Please see the Office action, *e.g.*, page 10, lines 8 to 13.

Applicant respectfully avers that the Examiner has not met his or her initial burden to establish a reasonable basis to question the enablement provided for the claimed invention, and specifically address, below, how the art used to support the Office's enablement rejection is not sufficient to rebut the presumptively enabled specification. None of the cited references,

individually or in their totality, are sufficient to rebut the presumption of enablement. None of these references are directed to whether, or not, screening a large number of nucleic acid or phytase variants (of an exemplary nucleic acid or phytase of the invention) would have constituted undue experimentation to one skilled in the art at the time of the invention.

Broun *et al.*(1998) Science 282:1315-1317, shows that a small number of amino acid reside changes in the catalytic site of a family of structurally related enzymes can result in a change in activity (in particular, Broun found that as few as four amino acid substitutions can convert an oleate 12-desaturase to a hydroxylase and as few as six result in conversion of a hydroxylase to a desaturase). However, in Broun, there is no discussion regarding the merits or difficulties of protocols that screen for enzyme activity, *e.g.*, screen for enzyme activity in polypeptides having at least one conservative amino acid substitution in an exemplary phytase. In fact, it appears that Broun considered screening for enzyme activity in their enzyme variants a routine process. There is no discussion on whether changes in non-catalytic site amino acid residues have any effect on enzyme activity. In fact, Broun's data suggest that most changes in an enzyme's amino acid sequence (*e.g.*, non-catalytic site amino acid residues) are not important in determining, or changing, its catalytic specificity.

Witkowski *et al.* (1999) Biochemistry 38:11643-11650, also showed that a small number of amino acid residue changes in the catalytic site of a family of structurally related enzymes can result in a change in activity. Witkowski noted that beta-ketoacyl synthases involved in the biosynthesis of fatty acids and polyketides exhibit extensive sequence similarity and share a common reaction mechanism. Interestingly, Witkowski also noted that multiple sequence alignments identified catalytic sites and provided the first clues about the possible identities of residues that play critical roles in catalysis. In fact, as with Broun, Witkowski's data suggest that most changes in an enzyme's amino acid sequence (*e.g.*, non-catalytic site amino acid residues) are not important in determining, or changing, its catalytic specificity. In Witkowski, there is no discussion regarding the merits or difficulties of protocols that screen for enzyme activity, *e.g.*, screen for enzyme activity in polypeptides having at least one conservative amino acid substitution in an exemplary phytase. It appears that Witkowski considered screening for enzyme activity a routine process.

Seffernick *et al.*(2001) J. of Bacteriol. 183:2405-2410, also shows that a small number of amino acid residue changes in the catalytic site of an enzyme can result in a change in activity. Seffernick compared a deaminase (melamine deaminase) with a hydrolase (atrazine chlorohydrolase, AtzA) and found that each enzyme consists of 475 amino acids and differs by only 9 amino acids. Seffernick opined that their data suggest that the 9 amino acid differences between melamine deaminase and AtzA represent a short evolutionary pathway connecting enzymes catalyzing physiologically relevant deamination and dehalogenation reactions. As with Broun and Witkowski, Seffernick's data suggest that most changes in an enzyme's amino acid sequence (*e.g.*, non-catalytic site amino acid residues) are not important in determining, or changing, its catalytic specificity. In Seffernick, there is no discussion regarding the merits or difficulties of protocols that screen for enzyme activity, *e.g.*, screen for enzyme activity in polypeptides having at least one conservative amino acid substitution in an exemplary phytase. It appears that Seffernick also considered screening for enzyme activity a routine process.

Applicant respectfully avers that none of these references, individually or in their totality, are sufficient to rebut the instant application's presumption of enablement. None of these references are directed to whether, or not, screening polypeptide sequence variants would have constituted undue experimentation to one skilled in the art at the time of the invention. In fact, because Broun, Witkowski and Seffernick's data suggest that most changes in an enzyme's amino acid sequence (*e.g.*, non-catalytic site amino acid residues) are not important in determining, or changing, its catalytic specificity, these references support the idea that most changes in an enzyme's amino acid sequence will result in little or no effect on its specificity or activity, and that one of skill in the art could easily target a minimum number of residues to generate a limited number of enzyme variants to generate desired enzyme variants. Accordingly, the Office has not established a reasonable basis to question the enablement provided for the claimed invention.

The Office must weigh all submitted evidence, including the specification and any new evidence supplied by applicant with the evidence and/or sound scientific reasoning previously presented in the rejection and decide whether the claimed invention is enabled. The Examiner should never make the determination based on personal opinion. The determination should always

be based on the weight of all the evidence. MPEP §2164.05, 8th edition, rev. 2, May 2004, pg 2100-190 to -191.

Applicants respectfully submit that the Examiner did not sufficiently consider and specifically address Dr. Short's previously submitted Rule 132 expert declaration regarding enablement, in which Dr. Short declared, *inter alia*, it would not have required any knowledge or guidance as to which are the specific structural elements, *e.g.*, amino acid residues, that correlate with phytase activity to create variants of the exemplary nucleic acid and test them for the expression of polypeptides or peptides having phytase activity, with reasons to doubt the objective truth of the statements contained therein. The evidence provided by Applicants need not be conclusive but merely convincing to one skilled in the art. MPEP 2164.05, 8th edition, rev. 2, May 2004, pg 2100-190 to -191. Applicant respectfully avers that their arguments, and Dr. Short's expert declaration, are sufficient to rebut any possible *prima facie* case of lack of enablement, i.e., Applicants have presented persuasive arguments that one skilled in the art would be able to make and use the claimed invention using the application as a guide.

An analysis of whether the claimed invention is supported by an enabling disclosure requires a determination of whether that disclosure contained sufficient information to enable one skilled in the pertinent art to make and use the claimed invention. Applicants respectfully aver that the specification enabled the skilled artisan at the time of the invention to identify, and make and use, the genus of phytases used in the claimed compositions and methods. As previously declared by Dr. Short, procedures for identifying polypeptides having phytase activity were conventional and routine in the art at the time of the invention. An exemplary assay for identifying polypeptides having phytase activity is described, inter alia, on page 35, section 6.1.5, of the specification. The specification also expressly sets forth which amino acid substitutions can be made to the exemplary SEQ ID NO:2 to make a variant phytase within the scope of the invention, i.e., one or more conservative amino acid substitutions to SEQ ID NO:2, including conservative amino acid substitutions comprising (a) a replacement, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile, (b) an interchange of the hydroxyl residues Ser and Thr, (c) an exchange of the acidic residues Asp and Glu, (d) a substitution between the amide residues Asn and Gln, (e) an exchange of the basic residues Lys and Arg, (f) a replacement among the aromatic residues Phe,

Tyr, or (g) a combination thereof. Also as previously declared by Dr. Short, the skilled artisan could have used routine screening to determine, with predicable positive results, which nucleic acids encoded for a phytase or with polypeptide variants had phytase activity.

The Patent Office also remains concerned that the specification does not teach a correlation between the structure of SEQ ID NO: 2 and phytase function (see, e.g., page 10, lines 1 to 4, of the office action). However, Applicants' respectfully aver that it was not necessary for the specification to describe any correlation between structure and function of phytases to enable one skilled in the art to make the genus of phytases of the invention. As declared by Dr. Short in a previous office action, one of ordinary skill in the art using the teaching of the specification would have been able to make and screen for nucleic acids that encode for one or more conservative amino acid substitutions to SEQ ID NO:2, including conservative amino acid substitutions comprising (a) a replacement, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile, (b) an interchange of the hydroxyl residues Ser and Thr, (c) an exchange of the acidic residues Asp and Glu, (d) a substitution between the amide residues Asn and Gln, (e) an exchange of the basic residues Lys and Arg, (f) a replacement among the aromatic residues Phe, Tyr, or (g) a combination thereof, and express those nucleic acids and using routine screening to determine, with predicable positive results, which of those nucleic acids encode for a polypeptide having phytase activity. Dr. Short declared that one of ordinary skill in the art using the teaching of the specification would have been able to make and use polypeptides of having conservative amino acid substitutions, or fragments of SEQ ID NO:2 having phytase activity. Successful results, including the identification of polypeptides having various conservative amino acid substitutions and having phytase activity using routine screening methods, were predictable. Furthermore, Dr. Short declared that it would not have required any knowledge or guidance as to which are the specific structural elements, e.g., amino acid residues, that correlate with phytase activity to make and identify nucleic acids that encode for phytases having one or more conservative amino acid substitutions to SEQ ID NO:2, including conservative amino acid substitutions. Thus, making polypeptides having the conservative amino acid substitutions described in the specification based on an exemplary enzyme sequence and screening them for phytase activity under various conditions was a predictable art at the time of the invention.

While Applicants maintain that it was not necessary that one of ordinary skill in the art knew the correlation between the structure of SEQ ID NO: 2 and phytase function to enable the claimed invention, in fact, such structure-function information for phytases was available to one of ordinary skill in the art. As discussed above, attached is an alignment of the phytase SEQ ID NO:2 (designated "DVSA") compared to five phytases known in the art at the time of the invention. The alignment shows areas of conserved sequence between the phytases, with completely conserved sequence residues highlighted in yellow. The sequence alignment highlights the conserved phytase active site, RHGXRXP as described, e.g., by Wodzinski and Ullah, "Phytase", in Advantages in Applied Microbiology, vol. 42, 1996, Academic Press, Inc. (copy attached) (see also, Berka (1998) Applied and Environ. Biol. 64:4423-4427; Kerovuo (1998) Applied and Environ. Biol. 64:2079-2085) (copies attached). Accordingly, one of skill in the art at the time of the invention would have had sufficient knowledge of the structure-function relationship of phytases and phytase active sites to design variant phytase sequences without undue experimentation.

The Patent Office also remains concerned about the size of the genus of claimed nucleic acids (including the genus of nucleic acids used in the methods of the invention). To address the Office's concerns, the claimed genus (see new claims 47 to 76) is amended such that the claimed phytases are further limited such that their sequences must have at least about 50% sequence identity to SEQ ID NO:2.

In conclusion, Applicants respectfully submit that one of ordinary skill in the art using the teaching of the specification would have been able to make and use polypeptides of having conservative amino acid substitutions, or fragments of SEQ ID NO:2 having phytase activity. Successful results, including the identification of polypeptides having various conservative amino acid substitutions and having phytase activity using routine screening methods, were predictable. Accordingly, Applicants respectfully submit that the pending claims meet the enablement requirements under 35 U.S.C. §112, first paragraph. In light of the above remarks, Applicants respectfully submit that amended claims are fully enabled by and described in the specification to overcome the rejection based upon 35 U.S.C. §112, first paragraph.

Non-statutory Double Patenting

Claims 1 to 13, 16-27, 29 to 32 and 34 and new claims 35-46 stand provisionally rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-15, 17-26, and 91-94 of copending application number 10/430,356.

Applicants note that in copending application number 10/430,356, only claims 45, 46, 52, 53, 73, 87, 91, and 95-123 are currently pending.

Applicant wish to hold this issue in abeyance until such time claims are held allowable.

CONCLUSION

In view of the foregoing amendment and remarks, Applicants respectfully aver that the Examiner can properly withdraw the rejection of the pending claims under 35 U.S.C. §112, first and second paragraphs. In view of the above, claims in this application after entry of the instant amendment are believed to be in condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejections of the claims and to pass this application to issue.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 564462001802. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

As noted above, Applicants have requested a telephone conference with the undersigned representative to expedite prosecution of this application. After the Examiner has reviewed the instant response and amendment, please telephone the undersigned at 858 720 5133.

Dated: November 23, 2004

Respectfully submitted,

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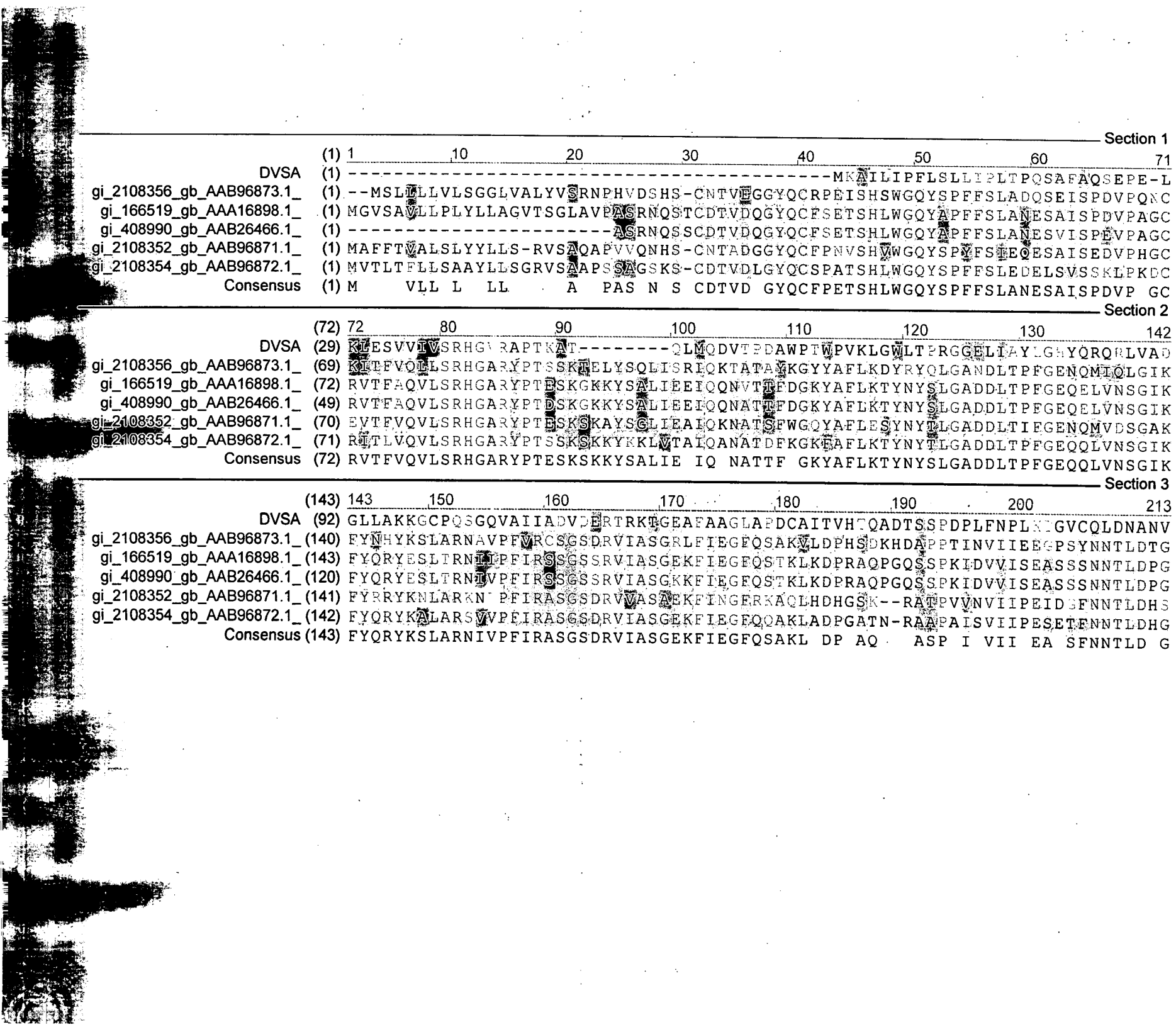
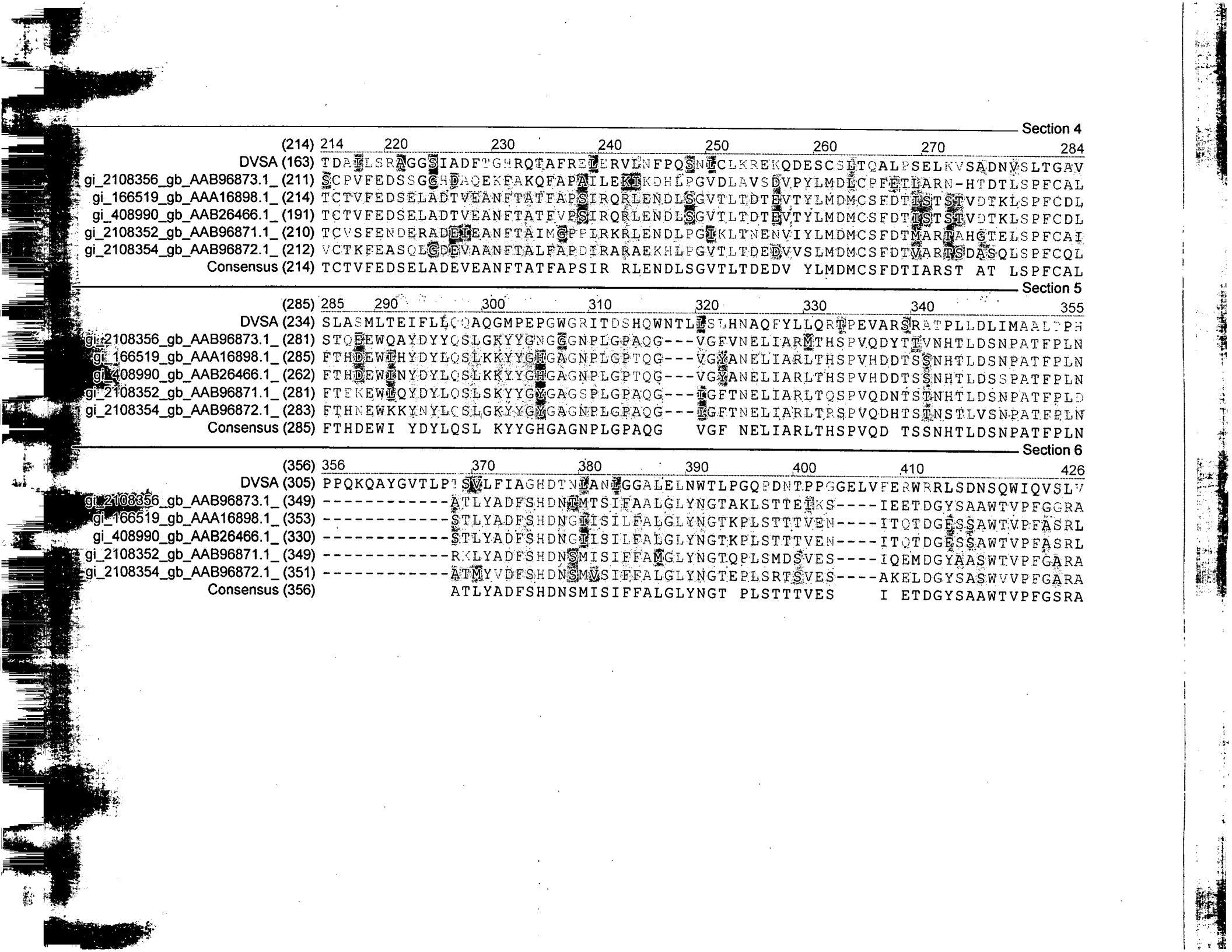

Section 1
(1) 1 10 20 30 40 50 60 71
DVSA (1) ----------MKAILIPFLSLLIPLTPQSAFAQSEPE-L
gi_2108356_gb_AAB96873.1_ (1) --MSLLLLVLSGGLVALYVSRNPHVDSHS-CNTVEGGYQCRPEISHSWGQYSPFFSLADQSEISPDVPQNC
gi_166519_gb_AAA16898.1_ (1) MGVSAVLLPLYLLAGVTSGLAVPASRNQSTCDTVDQGYQCFSETSHLWGQYAPFFSLANESAISPDVPAGC
gi_408990_gb_AAB26466.1_ (1) ----------ASRNQSSCDTVDQGYQCFSETSHLWGQYAPFFSLANESVISPEVPAGC
gi_2108352_gb_AAB96871.1_ (1) MAFFTVALSLYYLLS-RVSAQAPVVQNHS-CNTADGGYQCFPNVSHVWGQYSPYFSLEQESAISEDVPHGC
gi_2108354_gb_AAB96872.1_ (1) MVTLTFLLSAAYLLSGRVSAAPSSAGSKS-CDTVDLGYQCSPATSHLWGQYSPFFSLEDELSVSSKLPKDC
Consensus (1) M VLL L LL A PAS N S CDTVD GYQCFPETSHLWGQYSPFFSLANESAISPDVP GC
Section 2
(72) 72 80 90 100 110 120 130 142
DVSA (29) KLESVVIVSRHGVRAPTKAT--------QLMQDVTPDAWPTWPVKLGWLTPRGGELIAYLGHYQRQRLVAD
gi_2108356_gb_AAB96873.1_ (69) KLTFVQLLSRHGARYPTSSKTELYSQLISRIQKTATAYKGYYAFLKDYRYQLGANDLTPFGENQMIQLGIK
gi_166519_gb_AAA16898.1_ (72) RVTFAQVLSRHGARYPTESKGKKYSALIEEIQQNVTTFDGKYAFLKTYNYSLGADDLTPFGEQELVNSGIK
gi_408990_gb_AAB26466.1_ (49) RVTFAQVLSRHGARYPTDSKGKKYSALIEEIQQNATTFDGKYAFLKTYNYSLGADDLTPFGEQELVNSGIK
gi_2108352_gb_AAB96871.1_ (70) EVTFVQVLSRHGARYPTESKSKAYSGLIEAIQKNATSFWGQYAFLESYNYTLGADDLTIFGENQMVDSGAK
gi_2108354_gb_AAB96872.1_ (71) RITLVQVLSRHGARYPTSSKSKKYKKLVTALQANATDFKGKEAFLKTYNYTLGADDLTPFGEQQLVNSGIK
Consensus (72) RVTFVQVLSRHGARYPTESKSKKYSALIE IQ NATTF GKYAFLKTYNYSLGADDLTPFGEQQLVNSGIK
Section 3
(143) 143 150 160 170 180 190 200 213
DVSA (92) GLLAKKGCPQSGQVAIIADVDERTRKTGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNANV
gi_2108356_gb_AAB96873.1_ (140) FYNHYKSLARNAVPFVRCSGSDRVIASGRLFIEGFQSAKVLDPHSDKHDAPPTINVIIEESPSYNNTLDTG
gi_166519_gb_AAA16898.1_ (143) FYQRYESLTRNIVPFIRSSGSSRVIASGEKFIEGFQSTKLKDPRAQPGQSSPKIDVVISEASSSNNTLDPG
gi_408990_gb_AAB26466.1_ (120) FYQRYESLTRNIVPFIRSSGSSRVIASGKKFIEGFQSTKLKDPRAQPGQSSPKIDVVISEASSSNNTLDPG
gi_2108352_gb_AAB96871.1_ (141) FYRRYKNLARKNVPFIRASGSDRVVASAEKFINGFRKAQLHDHGSK--RATPVVNVIIPEIDSFNNTLDHS
gi_2108354_gb_AAB96872.1_ (142) FYQRYKALARSVVPFIRASGSDRVIASGEKFIEGFQQAKLADPGATN-RAAPAISVIIPESETFNNTLDHG
Consensus (143) FYQRYKSLARNIVPFIRASGSDRVIASGEKFIEGFQSAKL DP AQ ASP I VII EA SFNNTLD G


Section 4
(214) 214 220 230 240 250 260 270 284
DVSA (163) TDALLSRAGGSIADFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPSELKVSADNVSLTGAV
gi_2108356_gb_AAB96873.1_ (211) SCPVFEDSSGGHDAQEKFAKQFAPAILEKIKDHLPGVDLAVSDVPYLMDLCPFETLARN-HTDTLSPFCAL
gi_166519_gb_AAA16898.1_ (214) TCTVFEDSELADTVEANFTATFAPSIRQRLENDLSGVTLTDTEVTYLMDMCSFDTISTSTVDTKLSPFCDL
gi_408990_gb_AAB26466.1_ (191) TCTVFEDSELADTVEANFTATFVPSIRQRLENDLSGVTLTDTEVTYLMDMCSFDTISTSTVDTKLSPFCDL
gi_2108352_gb_AAB96871.1_ (210) TCVSFENDERADEIEANFTAIMGPPLRKRLENDLPGIKLTNENVIYLMDMCSFDTMARTAHGTELSPFCAI
gi_2108354_gb_AAB96872.1_ (212) VCTKFEASQLGDEVAANFTALFAPDIRARAEKHLPGVTLTDEDVVSLMDMCSFDTVARTSDASQLSPFCQL
Consensus (214) TCTVFEDSELADEVEANFTATFAPSIR RLENDLSGVTLTDEDV YLMDMCSFDTIARST AT LSPFCAL
Section 5
(285) 285 290 300 310 320 330 340 355
DVSA (234) SLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLISLHNAQFYLLQRTPEVARSRATPLLDLIMAALTPH
gi_2108356_gb_AAB96873.1_ (281) STQEEWQAYDYYQSLGKYYGNGGGNPLGPAQG---VGFVNELIARMTHSPVQDYTTVNHTLDSNPATFPLN
gi_166519_gb_AAA16898.1_ (285) FTHDEWTHYDYLQSLKKYYGHGAGNPLGPTQG---VGYANELIARLTHSPVHDDTSSNHTLDSNPATFPLN
gi_408990_gb_AAB26466.1_ (262) FTHDEWTNYDYLQSLKKYYGHGAGNPLGPTQG---VGYANELIARLTHSPVHDDTSSNHTLDSSPATFPLN
gi_2108352_gb_AAB96871.1_ (281) FTEKEWTQYDYLQSLSKYYGYGAGSPLGPAQG---IGFTNELIARLTQSPVQDNTSTNHTLDSNPATFPLD
gi_2108354_gb_AAB96872.1_ (283) FTHNEWKKYNYLQSLGKYYGYGAGNPLGPAQG---IGFTNELIARLTRSPVQDHTSTNSTLVSNPATFPLN
Consensus (285) FTHDEWI YDYLQSL KYYGHGAGNPLGPAQG VGF NELIARLTHSPVQD TSSNHTLDSNPATFPLN
Section 6
(356) 356 370 380 390 400 410 426
DVSA (305) PPQKQAYGVTLPTSVLFIAGHDTNLANLGGALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIQVSLV
gi_2108356_gb_AAB96873.1_ (349) ----------ATLYADFSHDNTMTSIFAALGLYNGTAKLSTTEIKS----IEETDGYSAAWTVPFGGRA
gi_166519_gb_AAA16898.1_ (353) ----------STLYADFSHDNGIISILFALGLYNGTKPLSTTTVEN----ITQTDGFSSAWTVPFASRL
gi_408990_gb_AAB26466.1_ (330) ----------STLYADFSHDNGIISILFALGLYNGTKPLSTTTVEN----ITQTDGFSSAWTVPFASRL
gi_2108352_gb_AAB96871.1_ (349) ----------RKLYADFSHDNSMISIFFAMGLYNGTQPLSMDSVES----IQEMDGYAASWTVPFGARA
gi_2108354_gb_AAB96872.1_ (351) ----------ATMYVDFSHDNSMVSIFFALGLYNGTEPLSRTSVES----AKELDGYSASWVVPFGARA
Consensus (356) ATLYADFSHDNSMISIFFALGLYNGT PLSTTTVES I ETDGYSAAWTVPFGSRA

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                    (427) 427                 440                 450                 460                 470                 480                 490
                   DVSA (376) EQTLQQMRDKTPLSLNTPPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL-------
gi_2108356_gb_AAB96873.1_ (404) YIEMMQCDDSDEPVVRVLVNDRVVPLHGCE-VDSLGRCKRDDFVRGLSFARQGGNWEGCVAASE
 gi_166519_gb_AAA16898.1_ (408) YVEMMQCQAEQEPLVRVLVNDRVVPLHGCP-IDALGRCTRDSFVRGLSFARSGGDWAECSA---
 gi_408990_gb_AAB26466.1_ (385) YVEMMQCQAEQEPLVRVLVNDRVVPLHGCP-VDALGRCTRDSFVRGLSFARSGGDWAE------
gi_2108352_gb_AAB96871.1_ (404) YFELMQCE-KKEPLVRVLVNDRVVPLHGCA-VDKFGRCTLDDWVEGLNFARSGGNWKTCETL--
gi_2108354_gb_AAB96872.1_ (406) YFETMQCKSEKEPLVRALINDRVVPLHGCD-VDKLGRCKLNDFVKGLSWARSGGNWGECFS---
                Consensus (427) YVEMMQC AE EPLVRVLVNDRVVPLHGCE VDALGRCTRDDFVRGLSFARSGGNWAECFA
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Biophysical Characterization of Fungal Phytases (*myo*-Inositol Hexakisphosphate Phosphohydrolases): Molecular Size, Glycosylation Pattern, and Engineering of Proteolytic Resistance

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ABSTRACT

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases) are found naturally in plants and microorganisms, particularly fungi. Interest in these enzymes has been stimulated by the fact that phytase supplements increase the availability of phosphorus in pig and poultry feed and thereby reduce environmental pollution due to excess phosphate excretion in areas where there is intensive livestock production. The wild-type phytases from six different fungi, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Emericella nidulans*, *Myceliophthora thermophila*, and *Talaromyces thermophilus*, were overexpressed in either filamentous fungi or yeasts and purified, and their biophysical properties were compared with those of a phytase from *Escherichia coli*. All of the phytases examined are monomeric proteins. While *E. coli* phytase is a nonglycosylated enzyme, the glycosylation patterns of the fungal phytases proved to be highly variable,

differing for individual phytases, for a given phytase produced in different expression systems, and for individual batches of a given phytase produced in a particular expression system. Whereas the extents of glycosylation were moderate when the fungal phytases were expressed in filamentous fungi, they were excessive when the phytases were expressed in yeasts. However, the different extents of glycosylation had no effect on the specific activity, the thermostability, or the refolding properties of individual phytases. When expressed in *A. niger*, several fungal phytases were susceptible to limited proteolysis by proteases present in the culture supernatant. N-terminal sequencing of the fragments revealed that cleavage invariably occurred at exposed loops on the surface of the molecule. Site-directed mutagenesis of *A. fumigatus* and *E. nidulans* phytases at the cleavage sites yielded mutants that were considerably more resistant to proteolytic attack. Therefore, engineering of exposed surface loops may be a strategy for improving phytase stability during feed processing and in the digestive tract.

INTRODUCTION

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Phytic acid (*myo*-inositol hexakisphosphate) is the major storage form of phosphorus in plants. In the context of human and animal nutrition, the following two aspects of phytic acid are critically important (18, 29): (i) monogastric animals have only low levels of phytate-degrading enzymes in their digestive tracts, and since phytic acid itself is not resorbed, feed for pigs and poultry commonly is supplemented with inorganic phosphate in order to meet the phosphorus requirements of these animals; and (ii) phytic acid is an antinutrient factor, since it forms complexes with proteins and a variety of metal ions and therefore decreases the dietary availability of these nutrients.

Because of these problems, there is considerable interest in phytate-degrading enzymes. The phytases (*myo*-inositol hexakisphosphate 3- and 6-phosphohydrolases; EC 3.1.3.8 and 3.1.3.26) are a subfamily of the histidine acid phosphatases (14) and are found naturally in plants and microorganisms, particularly fungi. As a class, the phytases have been rather poorly characterized biochemically (for a recent review see reference 29). In order to more clearly define this class of enzymes, several phytases of fungal origin have been cloned and overexpressed by workers in our company (14-16). In this paper we describe the purification of these fungal phytases, the structural and biophysical properties of these enzymes, and the results of a comparison with a prokaryotic phytase from *Escherichia coli*.

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Expression in *Aspergillus niger*. The DNA fragments encoding the *A. niger* CB (30), *Aspergillus terreus* 9A1 (GenBank accession no. U59805), *Aspergillus fumigatus* (GenBank accession no. U59804), *Emericella nidulans* (GenBank accession no. U59803), and *Myceliophthora thermophila* (GenBank accession no. U59806) phytases were ligated as 5' *Nco*I or *Bsp*HI (introduced sites at the ATG start codon) 3' blunt-ended fragments

downstream of the *glaA* promoter into the *Nco*I-*Eco*RV site of the expression vector, as described by Mitchell et al. (14) and Pasamontes et al. (15). Transformation of *A. niger* NW205 (*ura*$^-$ *arg*$^-$ *nic*$^-$; kindly provided by F. Buxton) and screening for phytase-producing transformants were also done as described previously (15).

The phytase of *A. niger* (Natuphos; GenBank accession no. Z16414) was a commercial preparation obtained from BASF (Ludwigshafen, Germany) and was purified to homogeneity by anion-exchange chromatography.

Expression in *Saccharomyces cerevisiae*. The phytase genes were cloned into pScer-Ro11, a 2μ-based vector harboring a shortened version of the *gap*(FL) promoter and the *pho5* terminator (12), as well as the *ura3* gene as a selection marker. The intronless phytase genes of *A. fumigatus*, *A. niger* CB, and *A. terreus* CBS were cloned as *Eco*RI-*Eco*RV fragments downstream of the *gap*(FL) promoter in the *Eco*RI-*Bam*HI blunt-ended expression cassette. The gene for *E. nidulans* phytase was cloned as an *Eco*RI fragment into the corresponding site of pScer-Ro11. *S. cerevisiae* YMR4 (*ura*$^-$ *his*$^-$ *leu*$^-$ *pho3*$^-$ *pho5*$^-$; kindly provided by M. Riederer) was used for transformation. Individual transformants were grown initially for 1 to 2 days in minimal medium. Phytase production was tested after subsequent culture for 2 to 3 days in YPD medium.

Expression in *Hansenula polymorpha*. The phytase genes (intronless) of *A. terreus* CBS (GenBank accession no. U60412), *A. fumigatus*, and *Talaromyces thermophilus* (GenBank accession no. U59802) were cloned as *Eco*RI fragments into the corresponding site of the *H. polymorpha* expression vector pFP (4) downstream of the formate dehydrogenase (FMD) promoter (9). The resulting plasmids were transformed into *H. polymorpha* RB11 (*ura*$^-$). About 300 to 400 transformants of each construct were inoculated into minimal medium (YNB containing 2% glucose). After several passages under selective pressure to force multiple integration of the expression plasmids into the genome of *H. polymorpha*, single stable clones were tested for phytase activity.

In order to obtain acceptable expression levels, the first 35 N-terminal amino acids of the *T. thermophilus* phytase were replaced by the amino acid sequence MGVFVVLLSIATLFGSTSGTALGPRGNHSKSCDTA$_{35}$ (the underlined amino acids originate from *A. terreus* CBS phytase and contain the signal sequence). Amino acids K$_{30}$SCDTA$_{35}$ were unrelated residues resulting from the cloning strategy used to replace the N terminus. Computer modelling of the chimeric *T. thermophilus* phytase suggested that modification of the first 16 amino acids of the mature protein was not likely to have an effect on the biochemical properties of the enzyme.

Protein purification. Independent of the expression system used, the culture broths (typically 500 to 1,000 ml) were centrifuged to remove the cells and were concentrated by ultrafiltration with Amicon 8400 cells (PM30 membranes; Grace AG, Wallisellen, Switzerland) and ultrafree-15 centrifugal filter devices (Biomax-30K; Millipore, Bedford, Mass.). The concentrates (typically 1.5 to 5 ml) were desalted with either Fast Desalting HR 10/10 or Sephadex G-25 Superfine columns (Pharmacia Biotech, Dübendorf, Switzerland); 10 mM sodium acetate (pH 5.0) was used as the elution buffer. The desalted *A. fumigatus* samples were directly loaded onto a 1.7-ml Poros HS/M cation-exchange chromatography

column (PerSeptive Biosystems, Framingham, Mass.). When the other phytases were expressed in *A. niger* or *H. polymorpha*, they were loaded onto a 1.7-ml Poros HQ/M anion-exchange chromatography column. During both anion-exchange and cation-exchange chromatography, phytase was eluted in pure form by using an optimized sodium chloride gradient.

All of the phytases expressed in *S. cerevisiae* (except *A. fumigatus* phytase) were brought to 2 M $(NH_4)_2SO_4$ after desalting and were loaded onto a 1-ml Butyl Sepharose 4 Fast Flow hydrophobic interaction chromatography column (Pharmacia Biotech). The enzymes were eluted with a linear 2 to 0 M $(NH_4)_2SO_4$ gradient in 10 mM sodium acetate (pH 5.0). The phytases eluted in the breakthrough and were concentrated and loaded onto a 120-ml Sephacryl S-300 gel permeation chromatography column (Pharmacia Biotech). They eluted as symmetrical peaks and were determined to be pure by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

***E. coli* phytase.** In order to clone the *appA* gene of *E. coli*, total DNA from *E. coli* M15 (25) was prepared as described by Davis et al. (3). A 500-ng portion of this DNA was used in a PCR performed with the following primers designed by using the *appA* sequence published by Dassa et al. (2) (GenBank accession no. M58708): appA I (5'-AAACATAT*TCATGA*AAGCGATCTTAATCCCA-3'), including an *Rca*I site (sequence in italics); and appA II (5'-ATATA*GGATCC*CAAACTGACAGCCGGTTATGCG-3'), including a *Bam*HI site (sequence in italics). The PCR was performed as described by the manufacturer (Expand High Fidelity PCR kit; Boehringer, Mannheim, Germany) by using a hybridization temperature of 55°C. The resulting PCR product was separated from the primers by using a PCR purification kit obtained from Qiagen (Hilden, Germany), was digested with *Bam*HI and *Rca*I, and was purified by agarose gel electrophoresis and a subsequent gel elution step (Qiaex II; Qiagen). The *appA* gene was ligated into the *Bam*HI and *Nco*I sites of the pBluescript II SK vector (Stratagene, La Jolla, Calif.). A DNA sequence analysis of the gene revealed two differences when it was compared to the sequence deposited by Dassa et al. (2). One of these differences was a change from A to G at position 620. This difference also affected the amino acid sequence of the *appA* gene product; the glycine at position 207 was replaced by aspartic acid. The second sequence difference was at position 984 (change from G to A) and was a silent mutation. The *appA* gene was transferred into the pQE60 expression vector (Qiagen) containing a C-terminal 6xHis tag and a short linker sequence (Gly-Ser-Arg-Ser-His-His-His-His-His-His) and was transformed into *E. coli* BL21 (Stratagene).

A 500-ml portion of Luria-Bertani medium containing 200 μg of ampicillin per ml and 30 μg of kanamycin per ml was inoculated with 8 ml of an overnight culture of strain BL21 harboring the *appA* expression plasmid. When the culture reached an optical density at 600 nm of 1.0, the cells were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated for an additional 5 h at 37°C with vigorous shaking. The cells were harvested by centrifugation at 4000 × *g* for 20 min, resuspended in 30 ml of sonication buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 20 mM imidazole, 1 mg of lysozyme per ml). The suspension was incubated on ice for 15 min before the cells were disrupted by sonication (Vibra Cell 72408; Bioblock Scientific, Illkirch, France) at 60% power by using 2.5-s bursts, 2.5-s cooling periods, and a net sonication time of 3 min. The supernatant was clarified by centrifugation at 10,000 × *g* for 15 min,

mixed with 8 ml of Ni^{2+}-agarose (50% resin suspension), equilibrated with sonication buffer, and incubated for 1 h on ice. Then the mixture was poured into a column and washed with 85 ml of sonication buffer. Phytase was eluted with 40 ml of 20 mM sodium acetate buffer (pH 4.5) containing 300 mM NaCl.

SDS-PAGE and IEF. SDS-PAGE was performed on 8 to 16% Tris-glycine gradient gels, and isoelectric focusing (IEF) was performed on IEF pH 3 to 7 or pH 3 to 10 gels (Novex, San Diego, Calif.). The gels were stained with colloidal Coomassie blue (Novex) or were semi-dry-blotted onto Immobilon P^{SQ} (polyvinylidene difluoride) membranes (Millipore) and then stained with amido black (naphthol blue black).

Gel permeation chromatography. The molecular sizes of the purified proteins were determined at room temperature by gel filtration performed with a calibrated Superdex 200 column (fast-protein liquid chromatography; Pharmacia Biotech). The elution buffer normally contained 50 mM sodium phosphate, 150 mM NaCl, 0.2 mM Na_2EDTA, 2 mM 2-mercaptoethanol, and 1 mM sodium azide (pH 7.2). In order to determine the effect of phosphate on the molecular size of *A. fumigatus* phytase, gel permeation chromatography was also performed with an elution buffer containing 100 mM sodium acetate (pH 5.0). The gel filtration column was calibrated for both elution buffers with high- and low-molecular-weight kits obtained from Pharmacia Biotech, which contained thyroglobulin (M_r, 749,000; Stokes radius, 85.0 Å), ferritin (M_r, 421,000; Stokes radius, 61.0 Å), catalase (M_r, 211,000; Stokes radius, 52.2 Å), aldolase (M_r, 163,000; Stokes radius, 48.1 Å), bovine serum albumin (M_r, 71,700; Stokes radius, 35.5 Å), ovalbumin (M_r, 45,700; Stokes radius, 30.5 Å), chymotrypsinogen A (M_r, 20,200; Stokes radius, 20.9 Å), and RNase A (M_r, 15,700; Stokes radius, 16.4 Å).

Analytical ultracentrifugation. Analytical ultracentrifugation was performed in 10 mM sodium acetate (pH 5.0) by using a model Optima XL-A ultracentrifuge (Beckman, Palo Alto, Calif.) equipped with a type An-60 Ti rotor and cells having standard double-sector Epon aluminum-filled centerpieces. The data were analyzed with the program DISCREEQ of Schuck (20), which is based on an iterative Marquardt procedure. The specific volume of a protein depends not only on the amino acid sequence but also on the extent and type of glycosylation. Since glycosylation of fungal phytases most likely is the high-mannose type of glycosylation, all sugars were assumed to be mannose. This assumption was a simplification but introduced only a small error into the M_r calculation.

Mass spectrometry. The peptides of interest obtained from trypsin digestion of *A. niger* phytase (Fig. 1) were analyzed by electrospray mass spectrometry. All analyses were performed in the positive ion mode with a triple quadrupole instrument (model API III; SCIEX, Concord, Ontario, Canada). Scans between *m/z* 400 and *m/z* 1600 were recorded with 0.2-average-mass-unit steps. *A. fumigatus* phytase was analyzed with a PerSeptive Biosystems Voyager Elite mass spectrometer equipped with a reflectron and delayed extraction. The ion acceleration voltage was 20 kV, and the results of 100 to 200 scans were averaged.

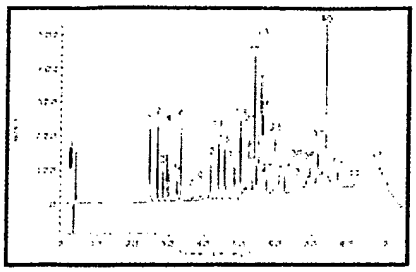

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FIG. 1. Separation of *A. niger* phytase fragments by reversed-phase HPLC after carboxymethylation and trypsin digestion of the protein. For experimental details see the text. AU, arbitrary units.

N-terminal sequencing and calculation of theoretical M_r and pI values. Automated Edman degradation of purified phytases was performed with a model ABD 494HT sequencer (Perkin-Elmer, Foster City, Calif.) with on-line microbore phenylthiohydantoin-amino acid detection. Theoretical M_r and pI values were calculated from the amino acid sequences of the native proteins (without a signal sequence) with the programs EditSeq and Protean from the Lasergene software package of DNASTAR Inc. (Madison, Wis.).

Proteolytic susceptibility of *A. fumigatus* phytase mutants. Purified *A. fumigatus* wild-type phytase and the *A. fumigatus* phytase mutants S126N and R125L/S126N were pretreated for 20 min at 90°C in order to inactivate potentially interfering traces of protease and then were renatured in the cold. Subsequently, they were incubated at 50°C at a concentration of 175 μg/ml in 10 mM sodium acetate (pH 5.0) with a 1:400-diluted 3-day culture supernatant of *A. niger* NW205 containing proteolytic activity. After 0, 20, 40, 60, 90, and 120 min of incubation, aliquots were subjected to SDS-PAGE or phytase activity was measured at 37°C. In control experiments, *A. fumigatus* wild-type phytase was incubated under the same conditions without NW205 culture supernatant or with NW205 culture supernatant that had been pretreated for 20 min at 90°C in order to inactivate the protease(s).

Identification of the glycosylation sites of *A. niger* phytase. *A. niger* phytase was S-carboxymethylated and digested with trypsin in 100 mM ammonium bicarbonate buffer (pH 8.0) at a phytase-to-protease ratio of 50:1 (wt/wt) for 18 h at 37°C. The reaction was stopped by adding 10% trifluoroacetic acid. The proteolytic fragments were separated at room temperature by reversed-phase high-performance liquid chromatography (HPLC) by using a C_{18} column (250 by 2.1 mm; Vydac, Hesperia, Calif.) and a linear 0 to 95% acetonitrile gradient in 0.1% trifluoroacetic acid (Fig. 1). All of the peptides were collected and analyzed by electrospray mass spectrometry. In addition, fractions 9, 17, 21, 27 to 30, 35, 37, and 43 to 45 (Fig. 1) were analyzed by Edman degradation. Assignments of glycosylated Asn residues were made on the basis of the absence of the phenylthiohydantoin derivative of Asn in the corresponding Edman cycles and by comparison of the peptide sequences with the predicted sequence of *A. niger* phytase.

Phytase activity measurements. Phytase activity was measured in an assay mixture containing 0.5% (~5 mM) phytic acid and 200 mM sodium acetate (pH 5.0). After 15 min of incubation at 37°C (or at temperatures between 37 and 90°C), the reaction was stopped by adding an equal volume of 15%

trichloroacetic acid. The liberated phosphate ions were quantified by mixing 100 μl of the assay mixture with 900 μl of H_2O and 1 ml of 0.6 M H_2SO_4-2% ascorbic acid-0.5% ammonium molybdate. After 20 min of incubation at 50°C, absorbance at 820 nm was measured. Standard solutions of potassium phosphate were used as a reference. One unit of phytase activity was defined as the amount of activity that liberates 1 μmol of phosphate per min at 37°C.

RESULTS

Purification of the phytases. Phytase genes from different fungal species were expressed in three different host organisms. *A. fumigatus* phytase is the only fungal phytase having an isoelectric point greater than 7.0 (Table 1). Since the pI of this phytase is considerably higher than the pIs of all of the extracellular proteins produced by the different expression strains, purification of *A. fumigatus* phytase was essentially a one-step procedure. As Fig. 2 shows, *A. fumigatus* phytase eluted as a symmetrical peak from a cation-exchange chromatography column. Probably because of acidic glycosylation, the pI of *A. fumigatus* phytase expressed in *H. polymorpha* was less than 4.0 (Fig. 3A). Nevertheless, cation-exchange chromatography could still be used for purification of this protein, although the capacity was much lower (data not shown).

TABLE 1. Isoelectric points of fungal and *E. coli* phytases

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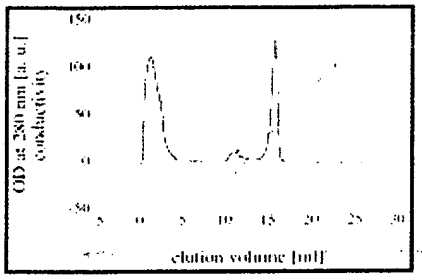

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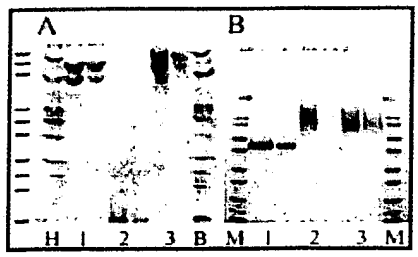
FIG. 2. Purification of *A. fumigatus* phytase by cation-exchange chromatography. An aliquot of a concentrated *A. niger* culture supernatant containing *A. fumigatus* phytase was loaded onto a 1.7-ml Poros HS/M cation-exchange chromatography column and eluted with a linear sodium chloride gradient. *A. fumigatus* phytase eluted as a symmetrical peak at an elution volume of approximately 15.5 ml. OD, optical density; a.u., arbitrary units.

FIG. 3. Extent of glycosylation and its effect on the isoelectric point of *A. fumigatus* phytase in different expression systems. (A) IEF pH 3 to 10 gel. (B) SDS-PAGE gel. Purified *A. fumigatus* phytase was expressed in *A. niger* (lanes 1), *H. polymorpha* (lanes 2), or *S. cerevisiae* (lanes 3)



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(two lanes with different protein concentrations were used for each expression system). Lane H, high-pI kit (Pharmacia Biotech); lane B, broad-pI kit (Pharmacia Biotech) (from top to bottom, pI 8.65, 8.45, 8.15, 7.35, 6.85, 6.55, 5.85, 5.20, 4.55, and 3.50); lanes M, Mark 12 molecular weight standard (Novex) containing myosin (M_r, 200,000), β-galactosidase (116,300), phosphorylase *b* (97,400), albumin (66,300), glutamic dehydrogenase (55,400), lactate dehydrogenase (36,500), carbonic anhydrase (31,000), trypsin inhibitor (21,500), lysozyme (14,400), and aprotinin (6,000).

The other fungal phytases expressed in *A. niger* or *H. polymorpha* had pI values that were less than 5.5 and, accordingly, could be purified by anion-exchange chromatography (data not shown). Although the high level of glycosylation associated with expression in *S. cerevisiae* had no effect on the pI of *A. niger* CB, *A. terreus* CBS, or *E. nidulans* phytase (data not shown), all attempts to bind these proteins to anion-exchange chromatography columns failed. Consequently, we used an alternative approach, in which these proteins were subjected to hydrophobic interaction chromatography followed by preparative gel permeation chromatography, which yielded homogeneous products (data not shown).

N-terminal sequences. In no instance did the N terminus of the mature fungal phytase that was determined experimentally (Table 2) correspond to the N terminus that was predicted on the basis of the von Heijne rules (26, 27). This finding is surprising and contrasts with observations made with other acid phosphatases (data not shown). Whether this deviation from the theoretical results is an inherent property of phytases or whether the initial processing conformed to the von Heijne rules but was followed by additional proteolytic cleavage could not be determined in this study. However, the latter possibility was supported by the finding that the *A. niger* NRRL 3135 (24), *A. niger* CB, *A. terreus* CBS, and *E. nidulans* phytases had multiple N-terminal sequences (Table 2). In the case of *E. coli* phytase, our sequence data agree with previously published N-terminal sequence data for the protein (2, 6). However, the amount of amino acid recovered in the Edman cycles was small compared with the amount of purified protein applied, suggesting that a large proportion of *E. coli* phytase is N-terminally blocked.

TABLE 2. N-terminal sequences of fungal and *E. coli* phytases

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Biophysical properties of fungal phytases are not affected by different extents of glycosylation. *A. fumigatus* phytase was expressed in three different expression systems, *A. niger*, *H. polymorpha*, and *S. cerevisiae*. In all of these expression systems, the protein was glycosylated, although to different extents (Fig. 3B). While glycosylation was moderate in *A. niger*, it was excessive and highly variable in *H. polymorpha* and *S. cerevisiae*, as indicated by the broad bands spanning M_rs ranging from 75,000 to 160,000. Different extents of glycosylation were observed even with different batches of *A. fumigatus*

phytase expressed in the same expression system (Table 3).

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TABLE 3. Molecular sizes of fungal and *E. coli* phytases as determined experimentally or by using the amino acid sequences of the mature proteins

When the protein was expressed in *A. niger* and *S. cerevisiae*, the different extents of glycosylation either had no or only a minor effect on the pI of the protein, suggesting that glycosylation was neutral (Fig. 3A). On the other hand, the pI of *A. fumigatus* phytase expressed in *H. polymorpha* was less than 4.0. Since deglycosylation with endoglycosidase F1 resulted in a pI of >8.0 (data not shown), the latter finding must have been due to acidic glycosylation. Remarkably, the different extents and patterns of glycosylation had no significant effect on the specific activity of the enzyme (31).

Virtually identical observations were made with *A. niger* CB, *A. terreus* CBS, and *E. nidulans* phytases which were also produced in different expression systems (data not shown).

Different extents of glycosylation may have an impact on the structure, stability, and function of proteins (11, 19, 28). In order to determine whether the extent of glycosylation had an effect on phytase stability, the activities of *A. fumigatus* phytase expressed in *A. niger*, *H. polymorpha*, or *S. cerevisiae* (Fig. 4A) and the activities of *A. niger* CB phytase expressed in *A. niger* or *S. cerevisiae* (Fig. 4B) were measured at a range of temperatures between 37 and 90°C. Evidently, the different extents of glycosylation had no effect on the thermostabilities of these two phytases. In addition, *A. niger* CB phytase expressed in either *A. niger* or *S. cerevisiae* was incubated for 20 min at temperatures between 37 and 90°C and then was incubated for 1 h at 4°C in order to allow (partial) refolding of the heat-denatured protein. Subsequent activity measurements at 37°C (Fig. 4C) revealed that the different extents of glycosylation had no effect on the refolding properties.

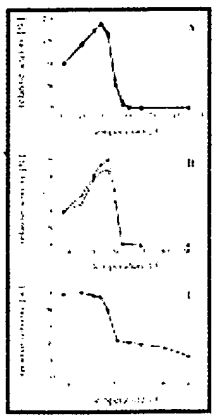

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FIG. 4. Different extents of glycosylation do not affect the thermostability and refolding of phytase. The enzymatic activity of *A. fumigatus* phytase (A) expressed in *A. niger* (□), *H. polymorpha* (•), or *S. cerevisiae* (▲) and the enzymatic activity of *A. niger* CB phytase (B) expressed in *A. niger* (□) or *S. cerevisiae* (▲) were measured at a series of temperatures between 37 and 90°C. (C) *A. niger* CB phytase expressed in *A. niger* (□) or *S. cerevisiae* (▲) was incubated for 20 min at 37, 45, 50, 53, 56, 60, 65, 70, 80, or 90°C and then for 1 h at 4°C. Subsequently, phytase activity was measured at 37°C. It is evident that the different extents of glycosylation had no or only minor effects on the thermostability and refolding properties of the phytases.

Fungal phytases are monomeric proteins. The molecular sizes of the phytases were determined by SDS-PAGE, analytical ultracentrifugation, gel permeation chromatography, and mass spectrometry and were calculated theoretically by using the amino acid sequences of the mature proteins (Table 3). While SDS-PAGE, mass spectrometry, and amino acid sequence determinations provided the M_r of the protomers, analytical ultracentrifugation and gel permeation chromatography provided values for the native proteins. The results obtained by the different methods agreed closely, and the data showed that all of the phytases investigated are monomeric proteins. There was no indication of higher oligomeric forms of phytase in any experiment. This interpretation is consistent with the results of an analysis of the three-dimensional structure, which also showed that the *A. niger* phytase is a monomeric protein (13).

The difference between the M_r determined by SDS-PAGE and the M_r calculated by using the amino acid sequence is an indication of the extent of glycosylation of a protein. In the examples listed above, the extent of glycosylation ranged from 20 to 65% of the total M_r, but it may be even higher. While mass spectrometry of *E. coli* phytase was straightforward, a broad, diffuse peak was obtained for *A. fumigatus* phytase. This finding is consistent with the difficulties encountered with mass spectrometry of highly glycosylated proteins.

For all of the glycosylated phytases, gel permeation chromatography consistently gave a higher M_r than expected. The difference between the values determined by gel filtration and SDS-PAGE (or analytical ultracentrifugation) increased with the extent of glycosylation, which was calculated from the difference between the M_r obtained by SDS-PAGE (or analytical ultracentrifugation) and the M_r obtained by amino acid sequence analysis (Fig. 5). Therefore, the higher M_rs obtained by gel filtration are most likely artifacts due to glycosylation. Similar observations have previously been made for Sephadex columns, in which glycoproteins also eluted earlier than expected (1).

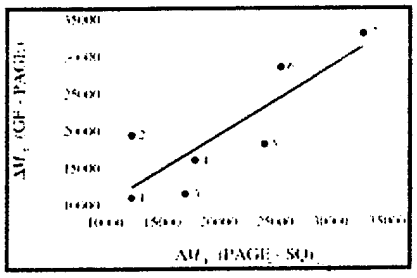

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FIG. 5. Gel filtration artifacts due to protein glycosylation. Gel permeation chromatography of the glycosylated phytases resulted in higher M_rs than expected on the basis of SDS-PAGE, mass spectrometry, or analytical ultracentrifugation data. The overestimation, as expressed by the difference between the M_rs obtained by gel filtration and SDS-PAGE (y axis), increased with the extent of protein glycosylation, as expressed by the difference between the M_rs obtained by SDS-PAGE and amino acid sequence analysis (x axis). The regression line has the equation $y = 904.1 + 0.9203x$ and an R value of 0.837. The data were obtained from Table 3. Data point 1, *M. thermophila* phytase; data point 2, *A. terreus* 9A1 phytase; data point 3, *E. nidulans* phytase; data point 4, *A. niger* phytase (Natuphos); data point 5, *A. fumigatus* phytase; data point 6, *A. niger* CB phytase; data point 7, *A. terreus* CBS phytase. GF, gel filtration; SQ, sequence analysis.

Isoelectric points. The isoelectric points of the different phytases were calculated by using the amino acid sequences of the mature proteins (without signal sequences) and were determined experimentally by IEF (Table 1). In general, there was a good correlation between the calculated and observed pI values, and the maximum difference was 1.28 pH units. This suggests that glycosylation in most instances either had no effect or had only a minor effect on the pI of the protein. The only exceptions were phytases expressed in *H. polymorpha*, in which a pronounced shift to acidic pI values and considerable pI heterogeneity were observed (Fig. 3A). As Table 1 shows, *A. fumigatus* phytase is peculiar in that it has a much higher pI than all of the other fungal phytases. Since the pI of this phytase also is higher than the pI values of most extracellular proteins produced by the expression strains used, *A. fumigatus* phytase is particularly suited to easy and efficient purification on an industrial scale. Because of the differences in pI values between individual phytases or for a single phytase expressed in different production hosts, different strategies had to be used for protein purification (see above).

Site-directed mutagenesis of surface-exposed cleavage sites decreases the proteolytic susceptibility of fungal phytases. When expressed in *A. niger* and stored as concentrated culture supernatants at 4°C, the phytases from *A. fumigatus*, *E. nidulans*, *A. terreus* 9A1, and *M. thermophila* had a tendency to undergo proteolytic degradation (data not shown). The results of N-terminal sequencing of the fragments (V_{153}VPFIRASGS for *A. fumigatus* phytase, R_{187}ATPVVNV and A_{188}TPVVNV for *E. nidulans* phytase, XP_{192}SPRVDVAI for *A. terreus* 9A1 phytase, and G_{282}RPLSPFXR for *M. thermophila* phytase) suggested that cleavage occurred between amino acids S-152 and V-153, K-186 and R-187 or R-187 and A-188, H-190 and Q-191, and N-281 and G-282, respectively. A comparison with the three-dimensional structure of *A. niger* phytase (13) and homology modelling of the other phytases (23a) revealed that all of the cleavage sites occur within surface-exposed loop structures or turns and are therefore accessible to proteases. While limited proteolysis of the *A. fumigatus* phytase between amino acids 152 and 153 was associated with pronounced or even complete inactivation of the enzyme (Fig. 6B), cleavage of the *E. nidulans* phytase between amino acids 186 and 187 or between amino acids 187 and 188 seemingly had no effect on the specific activity (data not shown). The two proteolytic fragments of both phytases remained associated with each other, as shown by gel permeation chromatography, as well as by copurification (data not shown). Site-directed mutagenesis at the protease-sensitive sites of *A. fumigatus* phytase (S152N and R151L/S152N) (Fig. 6B) and *E. nidulans* phytase (K186G/R187Q) (data not shown) yielded mutant proteins whose susceptibility to proteolysis was reduced considerably. On the other hand, the R151L and S152N mutations had no effect on the specific activity of *A. fumigatus* phytase, which was 20.3, 20.5, and 21.2 U/mg after heat pretreatment for the wild-type, the R151L/S152N, and the S152N enzymes, respectively.

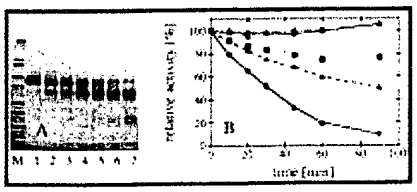

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FIG. 6. Proteolytic susceptibility of *A. fumigatus* phytase expressed in *A. niger*. (A) Purified *A. fumigatus* phytase was incubated at 50°C with a diluted *A. niger* NW205 culture supernatant containing proteolytic activity. After 0, 10, 20, 30, 45, 60, and 90 min of incubation, aliquots were subjected to SDS-PAGE (lanes 1 to 7, respectively). Lane M contained the Mark 12 molecular weight standard (see the legend to Fig. 3). (B) *A. fumigatus* wild-type phytase (•),

A. fumigatus S126N (■), and *A. fumigatus* R125L/S126N (△) were incubated with diluted NW205 culture supernatant at 50°C for 0, 10, 20, 30, 45, 60, and 90 min, and then phytase activity was measured at 37°C. As controls, *A. fumigatus* wild-type phytase was incubated under the same conditions without NW205 culture supernatant (○) or with NW205 culture supernatant that had been pretreated for 20 min at 90° C in order to inactivate the protease(s) (▲). The decreased rate of inactivation of the mutants was paralleled by much slower accumulation of degradation products on SDS-PAGE gels (data not shown).

In contrast to expression in *A. niger*, problems of proteolytic degradation were not encountered when the phytases were expressed in *H. polymorpha* (data not shown).

Identification of the glycosylation sites of *A. niger* phytase. *A. niger* phytase has 10 potential sites for N-linked glycosylation of the Asn-X-Ser/Thr type; these sites occur at residues 27, 59, 105, 120, 207, 230, 339, 352, 376, and 388, all of which are exposed on the surface of the molecule, as determined by an examination of the three-dimensional structure (13). In order to identify which of these residues are actually glycosylated in the mature protein, *A. niger* phytase was reduced, carboxy-methylated, and digested with trypsin. The proteolytic fragments were separated by reversed-phase HPLC (Fig. 1) and analyzed by electrospray mass spectrometry and Edman sequencing. Asn residues 27 (four peptides analyzed), 105 (one peptide analyzed), 207 (two peptides analyzed), 230 (two peptides analyzed), 339 (one peptide analyzed), and 376 (three peptides analyzed) were glycosylated in all of the peptides analyzed. In contrast, Asn-59 and Asn-120 were both glycosylated in only one of the two peptides examined. No glycosylation was observed for Asn-352 and Asn-388 in the one and three peptides analyzed, respectively. The incomplete glycosylation of Asn residues 59 and 120 may be one of the reasons for the M_r heterogeneity of *A. niger* phytase.

DISCUSSION

Depending on the application, an enzyme in which there is commercial interest should fulfill a series of predefined quality criteria. In the case of phytase, which is added to animal feed to increase the availability of phosphorus, these criteria include high specific activity, broad substrate specificity, a broad pH optimum, and good stability during storage, feed pelleting, and passage through the digestive tract. Thermostability is a particularly important issue since feed pelleting is commonly performed at temperatures between 65 and 95°C. In order to obtain information which can be used for engineering improved phytases, the range of phytase properties that occur in nature was investigated in this study by biochemically and biophysically characterizing different wild-type phytases, most of which were fungal phytases.

A comparison of the molecular sizes obtained by analytical ultracentrifugation, gel permeation

chromatography, mass spectrometry, SDS-PAGE, and amino acid sequence analyses (Table 3), as well as by X-ray crystallography (13), clearly showed that all of the fungal phytases considered as well as *E. coli* phytase are monomeric proteins. This conclusion is consistent with the findings of Ullah and coworkers for fungal and soybean seed phytases (5; for a review see reference 29), of Greiner et al. (6, 7) for *E. coli* and *Klebsiella terrigena* phytases, and of Shimizu (22) for *Bacillus subtilis* phytase. On the other hand, the phytases of *A. terreus*, *Aspergillus oryzae*, *Schwanniomyces castellii*, and maize roots were reported to be homohexameric, heterotetrameric or dimeric proteins (10, 21, 23, 32). The proposed hexameric structure of *A. terreus* phytase is particularly surprising, since in our study there was no indication of oligomeric forms of *A. terreus* phytases isolated from two different strains. Since inorganic phosphate might act as an effector molecule for phytases and the phosphate normally present in the gel filtration buffer may therefore have caused dissociation of phytase oligomers into monomers, gel permeation chromatography was also performed in the absence of inorganic phosphate. Again, there was no indication of higher oligomeric forms (data not shown).

In the four conflicting examples mentioned above, the molecular sizes of the phytases were determined by gel filtration on Sephadex G-200, Sepharose S-200, and Sephacryl HR S300 (10, 21, 32) or by native PAGE (23). As shown previously (1) and in this study (Fig. 5), gel filtration of glycosylated proteins results in overestimates of the molecular sizes, and the error increases with the extent of glycosylation. Calculation of the M_r from gel filtration experiments is based on the assumption that the proteins have a compact globular shape. As indicated by Andrews (1), glycoproteins may "have more expanded structures [...], which may well be due to a greater hydration in solution of carbohydrate chains as compared with polypeptide chains." In conclusion, gel permeation chromatography and possibly also native PAGE should not be considered reliable techniques for determining the molecular sizes of fungal phytases and of (highly) glycosylated proteins in general. Most, if not all, phytases of fungi, bacteria, and plants are monomeric proteins, and conflicting conclusions should be regarded with caution at least until there is more convincing evidence that oligomeric forms occur.

We found that glycosylation of the phytases was highly variable. It differed not only among the three expression systems used (Fig. 2) but also among different batches of a phytase produced in the same expression system (Table 3). As shown by the analysis of the glycosylation pattern of *A. niger* phytase (Fig. 1), some of the heterogeneity is due to the fact that 2 of the 10 potential N-glycosylation sites, Asn-59 and Asn-120, are nonstoichiometrically modified. Remarkably, different extents of glycosylation, such as those obtained when a phytase was produced in different expression systems (Fig. 3), had no effect on phytase thermostability and refolding properties (Fig. 4). The importance of glycosylation for phytase structure and function is further questioned by the fact that only two potential N-glycosylation sites, Asn-207 and Asn-339, are conserved in all of the fungal phytases whose primary structures are known (16).

Glycosylation may have a number of effects on the properties of an enzyme. First, it may have an impact on the stability of a protein, or it may influence the catalytic properties. Second, in case of acidic carbohydrate modification, it may influence the pI of a protein (Fig. 3) and thereby change the behavior of the protein during purification. And third, by diverting metabolic energy, it may lower the level of expression of a phytase. With regard to basic research, it is noteworthy that heterogeneous glycosylation

may impede crystallization of a protein and therefore determination of its three-dimensional structure. Strategies for circumventing this problem by deglycosylating glycoproteins, particularly phytase, with recombinant fusion protein glycosidases have recently been described by Grueninger-Leitch et al. (8).

All of the fungal, bacterial, and plant phytases investigated so far have acidic pI values (5-7, 10, 22, 23; for a review see reference 29). The difference between the pI values for *E. coli* phytase determined previously (about 6) (6) and in the present investigation (7.4 to 7.5) is most probably due to the His_6 tag attached to the C terminus in our preparation. In any case, *A. fumigatus* phytase, which has a pI of 8.1 to 8.6, is by far the most basic wild-type phytase. Since the great majority of contaminating proteins secreted by the expression systems into the culture supernatants have acidic pI values, this property makes *A. fumigatus* phytase a particularly well-suited enzyme for straightforward purification on an industrial scale (Fig. 2).

Finally, some of the phytases in some of the expression systems which we used showed a tendency to slowly undergo limited proteolysis. Identification of the cleavage sites by N-terminal sequencing of the fragments revealed that cleavage invariably occurred at exposed loops on the surface of the molecule. Site-directed mutagenesis of *A. fumigatus* phytase (Fig. 6) and *E. nidulans* phytase (data not shown) at these cleavage sites in fact reduced the susceptibility to proteases. Engineering of surface-exposed loops may therefore be a promising strategy for improving the resistance of phytases to proteases present in the culture supernatant or in the digestive tract.

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FOOTNOTES

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Phytase

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I. Introduction

A. THIRTY+ YEARS OF BASIC AND APPLIED SCIENTIFIC RESEARCH NECESSARY TO GENERATE A PRODUCT

1. *Overview of Product Development*

The research on phytase (Fig. 1) spans 87 years from its discovery by Suzuki *et al.*, (1907) until its commercialization in Europe in 1993–1994 by Gist–brocades. Commercialization required not only a practical use and delivery of the enzyme but also the ability to produce the enzyme

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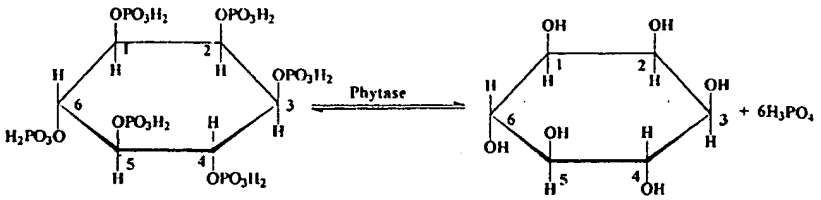

FIG. 1. Hydrolysis of phytic acid to inositol and phosphoric acid by phytase.

economically. The milestones are shown in Table I. Commercialization was not possible until methods were available to develop and produce high yields of the enzyme in microbial culture or in plants. Research to produce high yields of the enzyme was initiated whenever new techniques were discovered that had potential for progress. After the "new techniques" were applied to phytase production and it was determined that further progress was unlikely, research was suspended until newer techniques that appeared promising were developed. The story of commercialization of phytase is illustrative of the development of many products that are produced by microorganisms. They require fundamental discovery of new principles as well as applied long-term research. It is possible to use the phytase "story" to identify the principles that are germane for the commercial development of microbial products.

2. *Early Research*

The International Union of Biochemistry (1979) lists two phytases: a 3-phytase, EC 3.1.3.8, which hydrolyzes the ester bond at the 3 position of myoinositolhexakis phosphate to D-myoinositol 1,2,4,5,6-pentakisphosphate + orthophosphate and a 6-phytase, EC 3.1.3.26, which first hydrolyzes the 6-position of myo-inositolhexakis phosphate to D-myoinositol 1,2,3,4,5-pentakisphosphate + orthophosphate. Subsequent ester bonds in the substrate are hydrolyzed at different rates. The 6-phytase dephosphorylates phytic acid completely. However, the 3-phytase of at least one of the phytases described does not hydrolyze the phosphomono ester.

Posternak (1903) described phytic acid for the first time. Suzuki *et al.* (1907) were the first to describe the enzymatic activity of rice bran phytase and to prepare an extract that retained its activity. Dox and Golden (1911) demonstrated that aspergilli produce phytase. Early studies to identify organic phosphorus compounds in plant material were

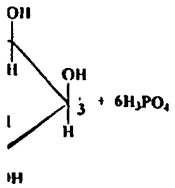

TABLE I

MILESTONES IN THE COMMERCIALIZATION OF PHYTASE

1903	Posternak—Describes phytic acid.
1907	Suzuki *et al.*—Describe and extract rice bran phytase.
1911	Dox and Golden—Demonstrate phytase in aspergilli.
1913	Plimmer and Anderson—Identify organic phosphorous compounds in plant material.
1914	Anderson—Determines the structure of phytic acid.
1959	Casida—Lists 20 soil fungi that have phytase activity.
1962–1971	**International Minerals and Chemicals initiates first commercial attempt to develop phytase as a product.**
1967	Ware and Shieh—Patent acid phytase.
1968	Shieh and Ware—Screen over 2000 isolates for phytase activity. Isolate *Aspergillus niger* NRRL 3135 syn *A. ficuum* produces phy A and phy B at the highest yield ever reported in a nongenetically modified strain.
1968	Nelson *et al.*—Feed phytase-treated soybean meal and document that hydrolyzed phytin is assimilated efficiently by broilers.
1971	Nelson *et al.*—Direct feeding of supplemental *A. niger* NRRL 3135 phytase to broilers in experimental and practical diets is titered.
1969	Shieh *et al.*—Partial purification characterization and regulation of *A. niger* NRRL 3135 phytase.
1984	**Southern Regional Research Center Agricultural Research Service, United States Department of Agriculture begin basic studies on phytase.**
1988	Ullah—Purified, characterized, and determined the partial amino acid sequence of *A. niger* NRRL 3135 phyA.
1993	Ehrlich *et al.*—Cloned and sequenced the gene for *A. niger* NRRL 3135 phyB.
1994	Ullah *et al.*—Cloned and sequenced the gene for *A. niger* NRRL 3135 metallo pH 6.0 acid phosphatase.
1987	**Alko Ltd. (Finland)(Pan Labs) initiates project to commercialize phytase.**
1993	Piddington *et al.*—Cloned and sequenced phytase from *A. niger* var. *awarmori*
1987	**Gist–brocades (The Netherlands) initiates project to commercialize phytase.**
1990	Simons *et al.*—Demonstration of efficacy of phytase in broilers and pigs.
1991	Van Gorcom *et al.*—Application for patent on the overproduction of phyA by cloned strains of *A. niger* NRRL 3135 and *A. niger* CBS 513.88 that has a glucoamylase promoter and in which synthesis is not controlled by levels of P. Extracellular yield of phyA increased by 50-fold.
1992	Ecological benefits of the use of phytase to abate phosphorous excretion by monogastric animals.
1993	Pen *et al.*—Expression of fungal phytase phyA in tobacco.
1994	Beudeker and Pen—Expression of fungal phytase phyA in canola (*Brassica napus*).

made by Plimmer (1913) and Anderson (1914a,b,c,d). They recognized the importance of phytin in corn soybean diets. Many early attempts to determine the structures of phytin were made. Eventually, the Anderson (1914d) structure, which is considered correct at this time, was developed (Fig. 1). Reddy *et al.* (1982) reviewed the structure of phytin and evaluated the data for each of the proposed structures.

It was recognized early that phytin was an effective chelating agent that bound metals and proteins in animal feedstuffs. As such, it is considered an antinutrient for monogastric animals. It binds calcium, iron, zinc, and protein. Most of the phosphorus of the phytic acid is not available to monogastric animals. A controversy as to whether phytin was hydrolyzed at significant rates in the intestines of rats, poultry, pigs, and man continued for many years. Many attempts were made to determine whether animals produced phytase in the intestine. Attempts were also made to feed live yeast that produced phytase to animals to aid in the hydrolysis of phytase *in vivo*. Results were unequivocal in the early research.

3. *Research at International Minerals and Chemicals (IMC) in the 1960s*

The first concentrated effort to make phytase a commercial product started in 1962 at International Minerals and Chemicals in Skokie, Illinois in which approximately 12 man years were expended on the project. The group in Microbial Biochemistry initiated the project to screen for microorganisms that produce phytase. Tsuong Rung Shieh screened over 2000 organisms for phytase activity. He isolated an organism from the soil in a flowerpot that produced the highest yields of phytase. Although many attempts have been made, no one has discovered a naturally occurring organism that produces more phytase in liquid culture. This organism was originally identified on the basis of its poor conidiagenesis, no known sexual cycle, and ability to produce oxalic acid rapidly as *Aspergillus ficuum* by Kenneth Raper at the University of Wisconsin. It was deposited as NRRL 3135. *Aspergillus ficuum* was previously placed in the taxonomic group *Aspergillus niger*. Centraalbureau voor Schimmelcultures designated NRRL 3135 as *A. niger* van Tieghem. Nuclear DNA reassociation studies suggest that *A. ficuum* is not a valid species and the *A. niger* designation should be conserved (Frisvad *et al.*, 1990; Peterson, 1992).

There are many references on phytase that refer to this organism. In this review, if we can identify with surety that this particular strain was used by the author, we will always refer tc it as *A. niger* NRRL 3135 regardless of what designation the author used.

Shieh *et al.* (1969) continued to develop the strain by optimizing the media and the conditions for production of the enzyme. The strain was

of special interest because one of the phytases (phyA) that it produced had two pH optima: one at pH 2.5 and one at pH 5.5. It also produced a second acid phosphatase with an optimum pH of 2.0 (phyB) that had activity on phytase. The intent of the project was to replace the feeding of inorganic phosphate by feeding phytase to monogastric animals. In 1962, the value of phytin-phosphorus (phytin-P) that was excreted by poultry was approximately $50 million.

It was necessary to test the feasibility of feeding phytase to broiler chicks to determine whether the enzyme would retain its activity when passed through the crop and intestine of the chicken. The pH optima of the enzyme approximated the pH of the chick digestive track and promised that the enzyme would be active in the crop of the chicken and at least partially in the intestine. Nelson *et al.*, (1968a) also tested the organism for its ability to grow in both moistened soybean meal and cotton seed meal. Feed studies were performed in chicks with meal that was pretreated with phytase. It was established (Nelson *et al.*, 1968a) that the phytin-P present in soybean meal was available to the chick and deposited in their bones if phytase was used to hydrolyze it.

The microbial biochemistry group at IMC scaled the process to the 114 L stage. Enough enzyme was produced and it was tested for use in monogastric animals by the animal nutrition group. The enzyme was fed to chicks and Nelson *et al.*, (1971) established that when phytase was fed to chicks, the phytin-P present in soybean meal and corn was made available and it was deposited in bones of the chick.

Unfortunately, the yields of phytase at this time were not high enough to produce a product that would be competitive with the feeding of inorganic phosphorus. The yield would have to be increased, by one estimate, about 250-fold just to break even. It was well known, at that time, that the phosphorus that was excreted by the chicks in the chicken manure would be mineralized by soil organisms and that it presented a pollution problem. Several attempts (media and applied genetics studies) were made to increase the yields of phytase to make it economic. However, they were unsuccessful and the project was terminated by IMC in 1968.

4. *Research at the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Southern Regional Research Center (SRRC) in the 1980s and 1990s*

In the 1980s, methodology became available that permitted the cloning of genes into microorganisms. Of special significance was the methodology that permitted the use of efficient promoters in gene constructs that increased yields significantly. This was recognized and in 1984 the technology that was developed at IMC was transferred to the

SRRC, ARS, and USDA. A research team, that expended approximately 16 man years, was formed at SRRC that isolated, characterized, and sequenced the phytases (phyA and phyB) and acid phosphatase produced by *A. niger* NRRL 3135. Their fundamental research provided the technical basis for the cloning studies and gene sequencing that followed at Gist–brocades, Pan Labs (Alko), and USDA.

5. *Research in Finland and The Netherlands*

At approximately the same time, Gist–brocades in the Netherlands initiated a project to abate pollution while trying to improve the yields of *A. niger* NRRL 3135. They expended approximately 40 man years. They cloned phyA along with an amyloglucosidase promoter into *A. niger* NRRL 3135. They increased the yield approximately 52-fold. They also cloned the enzyme along with a amyloglucosidase promoter and the *A. niger* NRRL 3135 leader sequence into *A. niger* CBS 513.88. They increased the yield of phytase by 1400 in one of the wild-type nonproducers (Van Gorcom *et al.*, 1991). In one of the trials listed, one of the cloned strains produced 270 μM/ml/min when the enzyme is assayed at 37°C. If one adjusts the reaction rate by multiplying by 3.7 to 58°C (Ullah, 1987), recalculates the unit to nKat, and divides by the specific activity of the pure enzyme (2100 nKat/mg protein), then approximately 7.9 g/liter is secreted into the medium by this bioengineered strain.

The nutrition group at Gist–brocades tested the enzyme extensively in poultry and swine. They have received approval in Austria, Belgium, Brazil, Bulgaria, Canada, Denmark, Germany, Norway, Finland, France, Greece, Ireland, Italy, Korea, Luxembourg, The Netherlands, Portugal, Spain, Switzerland, Taiwan, and the United Kingdom for use of the enzyme (Natuphos) as a feed additive. The FDA has approved a GRAS petition for use of phytase in food. Phytase is being marketed as a food additive in the United States as of January 1996.

Also, at approximately the same time, Alko in Finland began researching phytase in aspergilli. Their intended main use is in baking processes and animal feeds. They enlisted the group at Pan Labs (Bothell, WA) to increase the yield of the phyB enzyme by cloning procedures.

It is the purpose of this review not only to detail the events that were necessary to make phytase an economic reality but also to indicate that it is a combination of basic and applied research over long period of times that is often required to bring projects to fruition.

One can extrapolate the cost of performing this type of research by adding up the man years expended in research and multiplying a current cost factor per man year. The cost of discovering the fundamental required knowledge, developing a process for production of the en-

zyme, and demonstrating the efficacy (applications) and safety required not only the 68 man years documented previously but also the man years expended in various government, academic, and industrial research laboratories inherent in the research papers listed in the references that have direct application to solving the problem. It is noteworthy that it was the cooperation of industrial, government, and academic researchers that was necessary for capitalization of the results. It is the synergistic interactions of these, along with the generation of new methodology, that assures the introduction of new products in molecular biology and microbiology.

II. Importance of Phytic Acid and Hydrolysis Products

A. Role in the Plant

Phytic acid (myoinositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) (Fig. 1) is a major component of plant-derived food. It is the primary source of inositol and storage form of phosphorus in plant seeds that are used as animal feed ingredients (oilseed meals, cereal grains, and legumes) (Maga, 1982). Approximately 75% of the total phosphorus in cereals (Common, 1940), legumes (Nelson *et al.*, 1968b), and seeds (de Boland *et al.*, 1975; Erdman, 1979) exists as phytic acid phosphorus. Cottonseed meal, wheat, corn, rice, oats, soybean meal, and other plant-derived feedstuffs contain various amounts of phytate (Anderson, 1914a, b, c, d; Common, 1940; Erdman, 1979; Nelson *et al.*, 1968b; Rackis, 1974). Reddy *et al.*, (1982) reviewed the topic exhaustively.

Early speculation on the role of phytin-P in the plant centered on its use as a storage product. It was believed that large amounts of phosphorus were stored in the seed and that phosphorus was liberated on germination and incorporated into ATP. Recent studies have established the role of inositol phosphate intermediates in the transport of materials into the cell. Their role, especially that of inositol triphosphates, in transport as secondary messengers and in signal transduction in plant and animal cells is a very active area of research (Berridge, 1989, 1993; Berridge and Irvine, 1989; Morre *et al.*, (1990); Boitano *et al.*, 1992; Allbritton *et al.*, 1992; Iino and Endo, 1992).

There are many applications of phytic acid. Graf (1986) reviewed and listed 59 different applications including rust removal, prevention of dental caries, and its use as a hypocholesteromic agent. All of the applications center on the properties of phytic acid to chelate metals, interact electrostatically with proteins, its high affinity for hydroxyapatite, and interact with miscellaneous targets (Graf, 1986).

The applications of phytic acid and especially the inositol intermediates impinge on eventual applications of phytase. "Of broad nutritional and medical significance will be the preparation of myo-inositol, di, tri, tetra and pentaphosphate esters and their investigation of their interactions with different polyvalent cations" (Graf, 1986). Phytic acid or inositol intermediates have been implicated in starch digestibility and blood glucose response (Thompson, 1986), as an antioxidant (Graf *et al.*, 1987), in the lowering of cholesterol and triglycerides (Jariwalla *et al.*, 1990), in tumor formation (Shamsuddin *et al.*, 1988, 1989; Shamsuddin and Ullah, 1989; Ullah and Shamsuddin, 1990), in the treatment of Alzheimers disease (Sabin, 1988, 1989), in the treatment of Parkinson's disease (Sabin, 1992), and in the treatment of multiple sclerosis (Sabin, 1993). The preparation of these intermediates by immobilized phytase is provided under Section V, F

B. Role in Nutrition

1. *Phosphorous*

Ruminant animals sustain the microflora that enzymatically release inorganic phosphorus from phytic acid. However, phytic acid-phosphorus is essentially unavailable to monogastric animals: humans, chickens, and pigs (Nelson *et al.*, 1968a, b). They produce little or no phytase in the intestine and the phytin-P is excreted. An external source of phosphorus must be supplied in sufficient quantity to meet their daily mineral requirements (Nelson, 1967; Nelson *et al.*, 1968b).

Phytic acid that is present in the manure of these animals is enzymatically hydrolyzed by soil- and waterborne microorganisms. The released phosphorus is transported into rivers and lakes and can cause eutrophication in aquatic environments in which phosphorus is limiting. When it is introduced in high quantities, excessive algal growth and oxygen depletion ensue.

Phytic acid chelates divalent and trivalent cations. It binds Ca^{2+}, Zn^{2+}, Mg^{2+} and Fe^{3+} and trace minerals Mn^{2+}, Cu^{2+}, and Mo^{+} (Erdman, 1979; Maga, 1982; Nelson *et al.*, 1967, 1968b, Rackis 1974). It also binds proteins. Complexes may be formed with all of these constituents of the monogastric diet that renders them unavailable to the animal. Because all of these are required nutrients for monogastrics it influences the concentration that must be fed to the animals. Many have described phytin-P as an antinutrient factor. The antinutritive properties and its value as a possible phosphorus source, have stimulated researchers to develop

a method to remove phytic acid in a manner that is economically competitive with mineral supplementation.

One method for phytic acid removal is enzymatic treatment with phytase. Phytate phosphorus is decreased 81% in a mixture of ground maize, soybean meal, and wheat bran when treated with the native plant phytases (Zhu *et al.*, 1990). The tibia bone ash weights of the chicks fed the treated feed was higher than those of the control chicks (Zhu *et al.*, 1990). Although some feed ingredients contain native phytase, it is not a consistent source of the enzyme. The pH and temperature during feed processing may inactivate the enzyme (Maga, 1982; Nelson, 1967). The feeding of a phytase produced by a microorganism may ameliorate these problems.

III. Sources of Phytase

A. Plant Sources

Suzuki *et al.*, (1907) were the first investigators to make a preparation of phytase activity. They detected activity in rice and wheat bran. They also isolated inositol as a product of the reaction. The occurrence of phytase in germinating plants has been exhaustively reviewed by Reddy *et al.* (1982) and Gibson and Ullah (1990). They note that phytase has been isolated and/or characterized in cereals, such as triticale, wheat, corn, barley, and rice, and beans such as navy beans, mung beans, dwarf beans, and California small white beans.

Eeckhout and De Paepe (1994) measured phytase activity, phytin-P, and total P in 39 different samples of cereals, cereal by-products, oil meals, legume seeds, roots and tubers, and other plant byproducts. Only rye (85 nKat/g), triticale (28 nKat/g), wheat (20 nKat/g), and barley (10 nKat/g) were "rich" in phytase. Wheat byproducts, fine bran meal (76 nKat/g), or pellets (43 nKat/g), middlings (73 nKat/g), feed flour (56 nKat/g), and bran (49 nKat/g), also contained phytase. There was no correlation between the phytin-P content or total P with the phytase levels present in the plant-derived material. If one calculates the amount of phytase necessary to hydrolyze all of the phytin-P in a commercial diet and compares it with the amount of naturally occurring phytase in plants, it is evident that the commodity would have to be 40% of the diet to hydrolyze all of the phytin-P. This is not practical. Cloning of the *A. niger phyA* gene into plants that are used in practical diets along with efficient promoters circumvent the problem (see Section V,E.2).

Houde *et al.*, (1990) purified phytase from canola seed. However, the purified "phytase" from canola had 232 times more activity with pyrophosphate than it had with sodium phytate. The pH optimum of the plant enzymes range from 4.0 to 7.5. Most of the enzymes have a pH optimum between 5.0 and 5.6. They have an optimum temperature of 45 to 60°C and a K^m of 2.22×10^{-4} to 0.99×10^{-3} m*M*. The seeds of higher plants generally contain 6-phytase. Hayakawa *et al.*, (1990) presented data that rice bran F2 enzyme is able to dephosphorylate at the myoinositol 2 position. The levels of phytase in plants increase by several orders of magnitude during germination.

B. Bacterial Sources

Phytase has been detected in *Aerobacter aerogenes (Enterobacter aerogenes)* (Greaves *et al.*, 1967), *Bacillus subtilis* (Powar and Jagannathan, 1982), *Bacillus subtilis* (natto) N-77 (Shimizu, 1992), *Escherichia coli* (Greiner *et al.*, 1993), *Klebsiella aerogenes* (Tambe *et al.*, 1994), and *Pseudomonas* sp. (Irving and Cosgrove 1971) (Tables II, III, IV). The only bacterial organism that produces extracellular phytase is *B. subtilis*. When phytase is produced by bacteria, the yields are low and the pH optimum is neutral to alkaline that precludes their use as feed additives.

C. Fungal Sources

A number of surveys of microorganisms have been made for phytase production. The organisms that have been reported to produce extracellular phytase are shown in Table II. Shieh and Ware (1968) screened 2000 cultures from 68 soil samples and enrichments for phytase production. Howson and Davis (1983) surveyed 84 fungi from 25 species for phytase production. The incidence of phytase production is highest in aspergilli. The yields of phytase produced extracellularly are shown in Table III. Of all the organisms (plants, bacteria, and fungi) surveyed, *A. niger* NRRL 3135 produces the most active extracellular phytase in cornstarch (Shieh and Ware, 1968) and semisynthetic (Howson and Davis, 1983) media. It produces 110 nKat P/ml. *Aspergillus niger* NRRL 3135 produces two different phytases, one with pH optima at 5.5 and 2.5 and one with a pH optimum of 2.0. Later, these enzymes were designated phyA and phyB, respectively.

A variety of organisms produce phytase intracellularly (Table IV). A comparison of the properties of phytases that have been highly puri-

TABLE II

PRODUCTION OF EXTRACELLULAR PHYTASE BY MICROORGANISMS

Organism	No. of cultures tested	No producing acid phytase	Reference
Fungi			
Aspergillus spp. *3	3	3	Shieh and Ware (1968)
Aspergillus amstelodami	5	3	Howson and Davis (1983)
A. chevalieri	2	1	Howson and Davis (1983)
A. candidus	5	3	Howson and Davis (1983)
A. niger syn *A. ficuum*	1	1	Howson and Davis (1983)
A. flavus	10	2	Shieh and Ware (1968)
A. flavus	6	4	Howson and Davis (1983)
A. niger	1	1	Skowronski (1978)
A. niger	7	3	Howson and Davis (1983)
A. niger	22	21	Shieh and Ware (1968)
A. repens	4	3	Howson and Davis (1983)
A. sydowi	3	3	Howson and Davis (1983)
A. terreus	1	1	Yamada *et al.*, (1968)
A. terreus	2	1	Shieh and Ware (1968)
A. vesicolor	5	2	Howson and Davis (1983)
A. vesicolor	3	1	Shieh and Ware (1968)
A. wentii	1	1	Howson and Davis (1983)
Botrytis cinerea	5	2	Howson and Davis (1983)
Geotrichum candidum	1	1	Howson and Davis (1983)
Mucor spp.	37	1	Shieh and Ware (1968)
Mucor Priformis	4	2	Howson and Davis (1983)
M. Racemosus	5	4	Howson and Davis (1983)
Penicillium spp.	58	1	Shieh and Ware (1968)
Rhizopus oryzae	5	2	Howson and Davis (1983)
R. oligosporus	1	1	Howson and Davis (1983)
R. stolonifer	4	2	Howson and Davis (1983)
Saccharomyces cerevisiae	8	6	Howson and Davis (1983)
Schwanniomyces occidentalis syn. *S. castelli*	1	1	Segueilha *et al.* (1992)
Bacteria			
Bacillus subtilis	1	1	Powar and Jagannathan, (1982).
B. subtilis (natto) N-77	1	1	Shimizu (1992).

fied is shown in Table V. The extracellular phytases have molecular weights ranging from 36 to 85 kDa; a P_i of 4.0–6.3, a K_{cat} of 5.5–6209 per second, a K_m of 27–5000 micromol, an optimum pH of 2.5–7.5, and an optimum temperature of 35–63°C.

TABLE III

YIELDS OF EXTRACELLULAR PHYTASE PRODUCED IN CULTURE FILTRATES OR SUPERNATES

Organism	nKat/ml	μm P/min/ml	Reference
Fungi			
A. awamorii ATCC 11382	19.9	1.20	Shieh and Ware (1968)
A. awamorii ATCC 11358	28.6	1.72	Shieh and Ware (1968)
A. carbonarius NRRL 368	16	0.99	Shieh and Ware (1968)
A. carbonarius PCC 1040	13	0.78	Shieh and Ware (1968)
A. niger syn A. ficuum NRRL 3135	**110**	**6.62**	**Shieh and Ware (1968)**
A. niger syn *A. ficuum* WB 320	8.6	0.52	Shieh and Ware (1968)
A. niger syn *A. ficuum* WB 364	13	0.78	Shieh and Ware (1968)
A. niger syn *A. ficuum* WB 4016	9.46	0.57	Shieh and Ware (1968)
A. niger syn *A. ficuum* WB 4541	8.6	0.52	Shieh and Ware (1968)
A. niger syn *A. ficuum* WB 4781	10.4	0.625	Shieh and Ware (1968)
A. niger joponicus saito ATCC 1034	5.1	0.31	Shieh and Ware (1968)
A. niger ATCC 9142	18.1	1.09	Shieh and Ware (1968)
A. niger ATCC 10864	7.0	0.42	Shieh and Ware (1968)
A. niger var. *cinnamomeum* NRRL 348	12	0.73	Shieh and Ware (1968)
A. niger NRRL 326	6.0	0.36	Shieh and Ware (1968)
A. niger NRRL 330	7.0	0.42	Shieh and Ware (1968)
A. niger NRRL 337	4.3	0.26	Shieh and Ware (1968)
A. niger NRRL 372	8.6	0.52	Shieh and Ware (1968)
A. niger NRRL 4361	6.0	0.36	Shieh and Ware (1968)
A. niger van Tieghem 1	13.8	1.83	Shieh and Ware (1968)
A. niger K (soil isolate)	34.5	2.08	Shieh and Ware (1968)
A. niger x (soil isolate)	43.2	2.60	Shieh and Ware (1968)
A. satoi		0.94	Shieh and Ware (1968)
A. tubingensis NRRL 4875		1.25	Shieh and Ware (1968)
Bacteria			
Bacillus subtilis	4.0	0.24	Powar and Jagannathan (1982)
Bacillus subtilis (natto)	1.8	0.11	Shimizu (1992)

IV. Regulation of Phytase Synthesis

A. EFFECT OF SOURCE OF CORNSTARCH AND PHOSPHOROUS CONCENTRATION

Ware and Shieh (1967) related an invention in which the parameters for maximum acid phytase production by *A. niger* NRRL 3135 were listed. They discovered that the available inorganic phosphorus content of the medium controlled the synthesis of the enzyme. Maximum yields (113 nKat P/ml in shake flasks in 5 days) were obtained if the inorganic phosphorus content was controlled in the range of 0.0001% to about

TABLE IV

PHYTASE DETECTED IN CELLS OR WHOLE CULTURE

Aspergillus clavatus J239	Casida (1959)
A. flavipes Fla. A-14	Casida (1959)
A. flavus	Casida (1959)
A. nidulans QM-329	Casida (1959)
A. niger NRRL 67	Casida (1959)
A. niger P-330	Casida (1959)
A. niger	Dox and Golden (1911)
A. oryzae QM-228	Casida (1959)
A. phoenicus QM 329	Casida (1959)
A. repens QM-44c	Casida (1959)
A. terreus Fla. C-93	Casida (1959)
A. tamarii J1008	Casida (1959)
A. ustus QM-385	Casida (1959).
A. spp. 1*	Casida (1959)
A. spp. 2*	Casida (1959)
A. spp. 3*	Casida (1959)
A. spp. 4*	Casida (1959)
A. spp. 5*	Casida (1959)
Mucor spp.	Casida (1959)
Penicillium spp. P-320	Casida (1959)
Rhizopus spp.	Casida (1959)
Klebsiella aerogenes	Tambe *et al.* (1994)
Pseudomonas spp.	Irving and Cosgrove (1971)

TABLE V

PHYSICOCHEMICAL AND KINETIC PARAMETERS OF MICROBIAL PHYTASES AND ACID PHOSPHATASES

Enzyme	*Mr* (kDa)	P_i	K_{cat}	K_m (μm)	K_{cat}/K_m	Optimum pH	Optimum temperature
A. ficuum phyA[a]	85	4.5	348	27	1.29×10^7	5.0; 2.5	58
A. ficuum phyB[b]	68	4.0	628	103	6.1×10^6	2.5	63
A. ficuum, pH 6.0 APase[c]	85	4.9	260	200	1.3×10^6	6.0	63
E. coli phytase P2[d]	42	6.0	6209	130	4.77×10^7	4.5	35
E. coli, pH 2.5 APase[e]	45	6.3	2698	5000	5.4×10^5	2.5	37
B. subtilis[f]	38	6.3	5.5	500	1.1×10^4	6.0	60

[a] Ullah and Gibson (1987).
[b] Ullah and Cummins (1987b).
[c] Ullah and Cummins (1988b).
[d] Greiner *et al.* (1993).
[e] Dassa *et al.* (1980).
[f] Shimizu (1992).

0.005% by weight determined as phosphorus (Fig. 2). Shieh and Ware (1968) demonstrated the interaction of total phosphorus content with the carbon source in the medium and with the type of cornstarch that was used as the carbon source to phytase yield. The optimal conditions for phytase production were 0.4 mg/100 ml phosphorus and 8% w/v cornstarch (Hubinger). They noted that repression of phytase synthesis at high levels of P was a general phenomenon because it was observed with all molds and yeasts that produced phytase. The relative amounts of the pH 2.0 (phyB) and pH 5.5 phytase (phyA) differed depending on the amounts of inorganic phosphorus in the medium (Shieh *et al.*, 1969).

The regulatory effect of high P on phytase synthesis was confirmed by Howson and Davis (1983). Han and Gallagher (1987) also confirmed that high P concentrations inhibited phytase synthesis by *A. niger* NRRL 3135. When they used high amylose cornstarch (Hylon V National Cornstarch and Chemical Corp. Bridge Water, NJ), 1–5 mg P/100 ml was needed for maximum phytase production (44 nKat/ml in 7 days), whereas 8 mg P/100 ml was required for maximum cell growth. They also noted that the source of phosphorus played a role in the concentration of P required for maximum phytase synthesis. If the source of P was sparingly soluble, higher initial concentrations of P would produce the same levels of phytase. At 5 mg P/100 ml in the medium, growth was suboptimal but phytase production was optimal. It should be noted that the relative yields in their study are consistent with those of other studies. However, the yields of enzyme are low under the culture conditions that they used—oxygen starvation (50 ml in 125-ml flask at 200 rpm) and prolonged incubation (7–17 days).

Utt (1987) tested the effect of initial P concentration of eight different sources of commercial cornstarch on yields of phytase. Two treatment

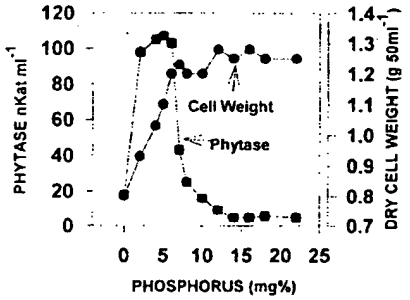

FIG. 2. Effect of initial phosphorus concentration in cornstarch medium on growth and production of phytase by *A. niger* NRRL 3135. From Shieh and Ware (1968).

methods were examined for their effectiveness in releasing P from cornstarch. Cornstarch preparations were autoclaved at 121°C for 15 min in basal fermentation medium and at 121°C for 24 hr in 2 *N* HCl. Significant differences between treatment methods occurred. Significantly higher levels of P were detected in the samples that were hydrolyzed for 24 hr as compared to those of the 15-min hydrolysis. Some commercial sources of cornstarch contain as much as 2.4 times as much P as other sources. One source had 8.25 μg P /g of cornstarch. In a medium that contains 80 g/liter of cornstarch, the level is significant (660 μg /liter) if one is attempting to control the level of P at 4 mg/liter. The levels of P in each of the samples were adjusted with P to 4 mg/liter and tested for phytase production. If total P content of the medium was the only factor in the cornstarch that affected yield, each of the conditions should have produced the same yield of phytase. Differences in the amount of phytase produced were as great as 5.2-fold depending on the specific cornstarch used. The source of cornstarch in which high yields of phytase yields (132 nKat/ml in shake flasks in 5 days) were produced consistently was Corn Products 3005 cornstarch (Fig. 3).

Gibson (1987) also confirmed that synthesis of phytase was controlled by the levels of phosphorus in the medium. She compared the production of enzyme from different sources of starch and speculated that the phosphoester linkage in some starch sources may be more resistant to cleavage than others, resulting in a low but steady supply of phosphorus, conversely some phosphoester linkages might be hydrolyzed at a

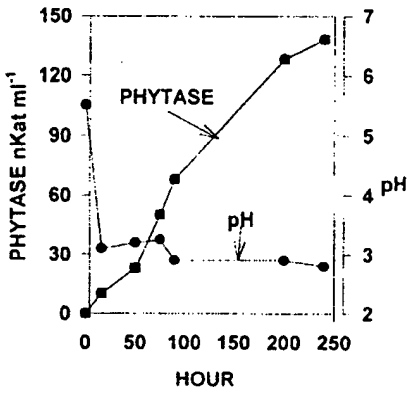

FIG. 3. Production of phytase by *A. niger* NRRL 3135 in 14-L fermenter with the following conditions: cornstarch medium (4 mg/100 ml total P) (Shieh and Ware, 1968); 28C; aeration, 0.18 liters/min; 15 psi; agitation, 350 rpm. From Ult (1987).

faster rate than others increasing the concentration of P in the medium and repressing synthesis of the enzyme. She also demonstrated that covalently bound P is present in Hylon V cornstarch and in potato starch as glucose-6-phosphate and glucose-3-phosphate. These phosphodextrins are effective substrates for the enzyme. If commercial cornstarch is used as a substrate in a medium to produce phytase using a strain of microorganism in which phytase synthesis is repressed by P, it is necessary to test that particular source of cornstarch for its effect on yield of the enzyme.

The question of phytase repression by high levels of P becomes moot in strains that are bioengineered for phytase production. Van Gorcom *et al.* (1991) removed the repression of phytase synthesis in genetically engineered *A. niger* NRRL 3135 and *A.* van Tieghem. Their work is discussed under Section V,E,2.

B. Effect of Medium Ingredients, Inoculum Size on Pellet Formation, and Phytase Yields

When a simple sugar, such as glucose or fructose is used as a sole source of carbon for phytase production by *A. niger* NRRL 3135, mycelial pellets are formed and the enzyme is made in low yields (Shieh and Ware, 1968). Han and Gallagher (1987) also observed these phenomena. However, if they used a medium containing surfactant (sodium oleate, 0.5% v/v), growth was dispersed and phyA yields were 4.7-fold higher than in controls in a 17-day incubation. The organism has also been propagated and phytase produced in submerged liquid fermentation and semisolid fermentation with cottonseed meal and soybean meal (Han, 1989; Han and Wilfred, 1988). The organism produces glucoamylase (Vandersall *et al.*, 1995) and α-galactosidase (Zapater *et al.*, 1990) extracellularly.

If either the inoculum size is too small, or if a relatively low viscosity medium (one devoid of cornstarch) is used, the organism forms pellets. Pellet formation may be minimized in shake flasks if at least 1×10^7 conidia are added per 50 milliliters of medium or if mass mycelial transfers are used [at least 10% (v/v) inoculum]. Pellet formation may also be minimized by the shearing action of the agitation system that breaks up aggregates of hyphae (Smith and Berry, 1974) and other factors such as pH, metal ion concentration, and complex or synthetic medium (Righelato, 1975). The property of lowered extracellular enzyme yields, when pellets are formed, appears to be a general characteristic of the organism. Reduced yields of α-galactosidase have also been observed when the organism formed pellets in a medium designed for production of that enzyme (R. J. Wodzinski, unpublished results).

C. Mutation Studies

Published results of mutation studies are sparse. Utt (1987) devised a medium that produced microcolonies and permitted large number of colonies per plate. However, the only mutants of *A. niger* NRRL 3135 isolated had limited increases in yield of phytase (17%). The increase in yield was probably due to an increase in phyB and not phyA. Chelius and Wodzinski (1994) isolated a mutant from the same wild-type strain that had a 3.3-fold increase in phyA activity, an 80% decrease in the pH 6.0 optimum acid phosphatase; and the same levels of phyB. The isolate may be a phytase catalytic mutant as well as an overproducer of phyA. The mutant approach is hampered by the lack of a powerful selection method that would differentiate between the phytases and phosphatases produced in a preliminary screen.

Classical mutation approaches for increasing yield of secreted proteins have value. Dunn-Coleman *et al.*, (1991) increased the yield of chymosin fivefold after the gene for the enzyme was cloned into *A. niger* var. *awamori* along with a glucose promoter. They noted an increase in secreted protein. A combination of cloning techniques with classical mutagenesis probably alters genes or properties of the secretion system that are not fully understood and that resist a purely rational approach to increasing yield.

V. Biochemistry of Phytase and Acid Phosphatases

A. Purification and Characterization

The activity of orthophosphoric monoester phosphohydrolase (acid optimum) has been described in a variety of species and tissues. To keep our focus on microbial enzymes, we will not describe in any detail the acid phosphatases produced in mammalian tissues. However, the mammalian acid phosphatase cannot be ignored altogether because there is sequence homology at the active site with the microbial phytase and acid phosphatases (Ullah and Dischinger, 1993b). Furthermore, an X-ray-deduced three-dimensional structure of recombinant rat prostatic acid phosphatase has been determined (Schneider *et al.*, 1993). The physicochemical and catalytic properties of phytases and acid phosphatases of microbial origin are summarized in Table V. *Aspergillus niger* NRRL 3135 phytase (pH 5.0 optimum phyA) and acid phosphatases [pH 2.5 optimum (phyB) and pH 6.0 optimum] are secreted proteins. The relative amounts of these proteins in the media are considerably higher when grown under phosphate starvation in starch media. One estimate from the purification tables (Ullah and Gibson, 1987; Ullah and Cummins, 1987b, 1988b) and the enzymes' K_{cat} indicated that approximately 50%

of the total secreted proteins are phyA, phyB, and the pH 6.0 optimum acid phosphatases. About 40% of the protein, from the enzyme activity data, was identified as pH 6.0 optimum acid phosphatase; phyA and phyB were about 5% each. It was estimated that to purify these enzymes to near homogeneity, only 5- to 25-fold purification of each enzyme was needed. The secreted glycoproteins of *A. niger* NRRL 3135 are stable for many months at 5°C and they lack intrinsic protease activity (A. Ullah, unpublished observation). A combination of these properties made it possible for investigators to purify these proteins at room temperature. The three enzymes are acidic proteins with P_i values below 5.0. Therefore, all three enzymes can be concentrated by passing the culture filtrate onto a strong cationic exchanger at pH 2.8 or below. Subsequent purification steps involve additional ion-exchange chromatography and chromatofocusing. Biochemical and kinetic characterizations were performed on the enzymes recovered from the terminal step. The determination of molecular mass of the fungal phytase and acid phosphatases was determined by either gel filtration or SDS–PAGE. The enzymes are microheterogeneous because they are differentially glycosylated. The molecular masses of the monomeric form of phyA, phyB, and pH 6.0 optimum acid phosphatase were estimated by SDS–PAGE to be 85, 65, and 85 kDa, respectively. The P_i's of the native fungal phytase, pH 2.5 optimum acid phosphatase, and pH 6.0 optimum acid phosphatase were estimated to be 4.5, 4.0, and 4.9, respectively. The hydrolysis pathway for phytate was only elucidated for the pH 5.0 optimum phytase (phyA); the enzyme was labeled 3-phytase (EC 3.1.3.8) (Ullah and Phillippy, 1988). The K^m and K_{cat} of fungal phytases for myoinositol hexa-, penta-, tetra-, and triphosphates have been reported (Ullah and Phillippy, 1994). The K_{cat}/K^m values indicate that the preferred substrate for both phyA and phyB is myoinositol hexaphosphate. Therefore, it is justifiable to designate these enzymes phytase.

An extracellular phytase and an extracellular acid phosphatase were purified from *Aspergillus oryzae* K1 (Shimizu, 1993). Unlike *A. niger* NRRL 3135 phytase and pH 6.0 optimum acid phosphatase, which are monomers, the active enzymes of *A. oryzae* are dimers. The molecular masses of *A. oryzae* phytase and acid phosphatase are 60 and 70 kDa, respectively. The P_i, temperature optima, and pH optima of *A. oryzae* enzymes are very similar to those of the *A. niger* NRRL 3135 phosphomonoesterases. A significant sequence homology is expected for this class of extracellular proteins.

An extracellular phytase from *B. subtilis* (natto) N-77 was purified 322-fold by gel filtration and DEAE chromatography (Shimizu, 1992). The molecular mass of the active monomeric form was judged to be 36 kDa by SDS–PAGE. The protein's P_i was estimated to be 6.25. Like the fungal en-

zymes, the optimum temperature for the bacterial phytase was about 60°C.

Two periplasmic phytases, P1 and P2, were purified from *E. coli* to near homogeneity (Greiner *et al.*, 1993). The active species were judged to be monomer with a molecular mass of 42 kDa. Both enzymes are very specific for phytate. The hydrolysis pathway for phytate was deduced for P2; the enzyme was identified as 6-phytase (EC 3.1.3.26). The pH optima for P2 was 4.5 for phytate and 3.5 for the synthetic substrate, *p*-nitrophenyl phosphate. Like *B. subtilis* phytase, the *E. coli* enzyme is also a weakly acidic protein with a P_i of 6.0. Both chemical and kinetic properties of P2 point to identity with an acid phosphatase (Dassa *et al.*, 1982).

A phytase was purified from yeast (Nayini and Markakis, 1984); however, purity and molecular data were not reported. The pH and temperature optima were 4.6 and 45°C, respectively. Although the enzyme can degrade a variety of inositol phosphatases—di-, tri-, tetra-, penta-, and hexaphosphate—inositol monophosphate was hydrolyzed by this enzyme at an accelerated rate. From the kinetic data, it is reasonable to class the enzyme as a myoinositol monophosphatase. It is notable that *A. niger* NRRL 3135 (phyA) shows poor substrate acceptability to myoinositol monophosphate. Therefore, the yeast enzyme is very different from the *A. niger* NRRL 3135 phytase in terms of substrate specificity.

Both *E. coli* and *A. niger* NRRL 3135 also produce acid phosphatases (Dassa *et al.*, 1980; Ullah and Cummins, 1988b). The bacterial enzyme resides at the periplasm; the enzyme has high affinity (K^m =2.7 μM) for *p*-nitrophenyl phosphate (Dassa *et al.*, 1980). The fungal enzyme, pH 6.0 optimum acid phosphatase, is a highly glycosylated extracellular metalloenzyme (Ullah *et al.*, 1994). This copper-containing phosphomonoesterase is a poor acceptor of myoinositol phosphates; based on K_{cat}/K_m ratio, phytate is hydrolyzed at an efficiency of 0.06% compared to phyA (100%). The K_{cat}/K_m ratio for the lower forms of myoinositol phosphates is one order of magnitude lower than that of hexaphosphate. The substrate accommodation data for the fungal pH 6.0 optimum acid phosphomonoesterase clearly suggest the enzyme to be an acid phosphatase.

B. Sequence Studies

The primary structure of *A. niger* NRRL 3135 phytase (phyA) was determined from both the cloned DNA (GenBank Accession No. M94550) and chemical sequencing (Ullah and Dischinger, 1993a). The sequence deduced from DNA is in full agreement with the chemically deduced protein sequence. Phytase sequence has also been deduced from the cloned DNA of *A. niger* strain van Tieghem (Van Hartingsveldt et al., 1993) and is identical. A third phytase sequence was obtained from *A. niger* var. *awamori* (GenBank Accession No. L02421) and revealed 97.2%

homology to *A. niger* NRRL 3135 phytase (Fig. 4). The substituted amino acids in *A. niger* var. *awamori* revealed that of a total of 13 substitutions only 1 at the penultimate C-terminal end was nonhomologous. The other 12 substitutions in *A. niger* var. *awamori* phytase were all conservative replacements predicting a very similar tertiary structure for both of the proteins. The primary structure of *A. niger* NRRL 3135 phyB and *A. niger* var. *awamori* phyB was elucidated from the cloned DNA and a partial sequence was verified by chemical sequencing (Ehrlich et al., 1993;

```
                10        20        30        40        50        60
AFphya MGVSAVLLPLYLLSGVTSGLAVPASRNQSSCDTVDQGYQCFSETSHLWGQYAPFFSLANE
       :::::::::::::.:::::::::::::::.::::::::::::::::::::::::::::::
ANphya MGVSAVLLPLYLLAGVTSGLAVPASRNQSTCDTVDQGYQCFSETSHLWGQYAPFFSLANE
                10        20        30        40        50        60

                70        80        90       100       110       120
AFphya SVISPEVPAGCRVTFAQVLSRHGARYPTDSKGKKYSALIEEIQQNATTFDGKYAFLKTYN
       :.:::.:::::::::::::::::::::::::.:::::::::::::::.:::::::::::::
ANphya SAISPDVPAGCRVTFAQVLSRHGARYPTESKGKKYSALIEEIQQNVTTFDGKYAFLKTYN
                70        80        90       100       110       120

               130       140       150       160       170       180
AFphya YSLGADDLTPFGEQELVNSGIKFYQRYESLTRNIVPFIRSSGSSRVIASGKKFIEGFQST
       ::::::::::::::::::::::::::::::::::::::.:::::::::::::::.::::::::
ANphya YSLGADDLTPFGEQELVNSGIKFYQRYESLTRNIIPFIRSSGSSRVIASGEKFIEGFQST
               130       140       150       160       170       180

               190       200       210       220       230       240
AFphya KLKDPRAQPGQSSPKIDVVISEASSSNNTLDPGTCTVFEDSELADTVEANFTATFVPSIR
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::.::::
ANphya KLKDPRAQPGQSSPKIDVVISEASSSNNTLDPGTCTVFEDSELADTVEANFTATFAPSIR
               190       200       210       220       230       240

               250       260       270       280       290       300
AFphya QRLENDLSGVTLTDTEVTYLMDMCSFDTISTSTVDTKLSPFCDLFTHDEWINYDYLQSLK
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::.::::::
ANphya QRLENDLSGVTLTDTEVTYLMDMCSFDTISTSTVDTKLSPFCDLFTHDEWIHYDYLQSLK
               250       260       270       280       290       300

               310       320       330       340       350       360
AFphya KYYGHGAGNPLGPTQGVGYANELIARLTHSPVHDDTSSNHTLDSSPATFPLNSTLYADFS
       ::::::::::::::::::::::::::::::::::::::::::::.:::::::::::::::::
ANphya KYYGHGAGNPLGPTQGVGYANELIARLTHSPVHDDTSSNHTLDSNPATFPLNSTLYADFS
               310       320       330       340       350       360

               370       380       390       400       410       420
AFphya HDNGIISILFALGLYNGTKPLSTTTVENITQTDGFSSAWTVPFASRLYVEMMQCQAEQEP
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
ANphya HDNGIISILFALGLYNGTKPLSTTTVENITQTDGFSSAWTVPFASRLYVEMMQCQAEQEP
               370       380       390       400       410       420

               430       440       450       460
AFphya LVRVLVNDRVVPLHGCPVDALGRCTRDSFVRGLSFARSGGDWAECFA
       ::::::::::::::::.:::::::::::::::::::::::::::::: :
ANphya LVRVLVNDRVVPLHGCPIDALGRCTRDSFVRGLSFARSGGDWAECSA
               430       440       450       460
```

FIG. 4 Comparison of deduced phytase sequence from cloned phyA DNA of *A. niger* NRRL 3135 (AFphyA) (GenBank Accession No. M94550) with cloned DNA of *A. niger* var *awamori* (ANphyA) (GenBank Accession No. L02421) (97.2% homology).

Piddington et al., 1993). The phyB from *A. niger* NRRL 3135 showed 99% homology to the corresponding protein from *A. niger* var. *awamori*. Surprisingly, phyA revealed only 23.1% homology to the phyB (Fig. 5). The primary structure of the bacterial phytases has not been deduced

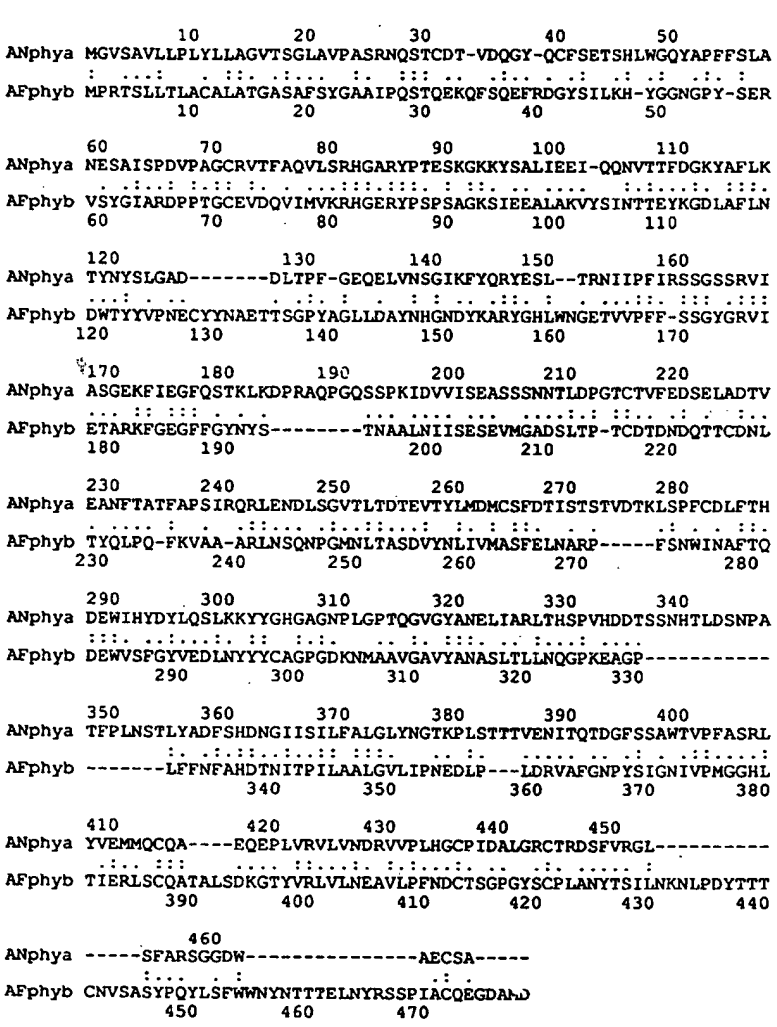

FIG. 5. Comparison of chemical sequenced phytase from cloned phyB DNA of *A. niger* NRRL 3135 (AFphyB) (Ehrlich et al., 1993) with cloned DNA of *A. niger* var *awamori* (ANphyA) (GenBank Accession No. L02421), (23.1% homology).

chemically; however, the primary structure of pH 2.5 optimum acid phosphatase from *E. coli* was deduced from the cloned DNA (Dassa et al., 1990). When sequence alignment was performed between a truncated *A. niger* NRRL 3135 phyB (-44 residues from the N terminus) and *E. coli* pH 2.5 acid phosphatase, the result showed only 15.1% homology (A. H. J. Ullah, unpublished data). Although phytase activity had been reported in yeast (Nayini and Markakis, 1984), the enzyme was not purified to homogeneity or any sequence reported to this date. However, acid phosphate genes *pho1* and *pho5* from yeast were cloned and sequenced (Elliott et al., 1986; Bajwa *et al.*, 1984). Protein sequence alignment of *A. niger* NRRL 3135 phyA and phyB with the deduced primary structure of *pho1* and *pho5* of yeast showed remarkable homology for cysteines, certain hydrophobic residues, and the active site residues (Ullah and Dischinger, 1995).

C. Active Site Determinations

The active site of phytases shows remarkable homology to the active site residues of the members of a particular class of acid phosphatase termed "histidine phosphatase" (Van Etten *et al.*, 1991; Ullah *et al.*, 1991). Chemical probing at the active site of human prostatic acid phosphatase suggested that an arginine residue is involved in catalysis (Van Etten, 1982). A similar observation was also made in *A. niger* NRRL 3135 (Ullah *et al.*, 1991). Sequence similarity search among diverse phosphate metabolizing enzymes, i.e., fructose 2,6-biphosphatase, phosphate glycerate mutase, and acid phosphatase, led to the identification of a tripeptidic region with the sequence RHG (Bazan *et al.*, 1989). When *A. niger* NRRL 3135 N-terminal amino acid sequence was compared with these enzymes, it was noted that the conserved tripeptidic region was also present not only in fungal phyA but also in the N-terminal region of phyB (Ullah and Dischinger, 1993b). Further chemical probing of the fungal phytase also suggested a sensitive histidine at the active site (Ullah and Dischinger, 1992). On close examination of the active site residues of phyA and phyB in *A. niger* NRRL 3135, pH 2.5 optimum acid phosphatase in *E. coli*, pho3 and pho5 gene products in yeast, human prostatic, and lysosomal acid phosphatase, it was observed that the most conserved sequence is RHGXRXP (Table VI). The acid phosphatases and phytases containing this active site motif in the N-terminal segment of the protein are grouped under "histidine phosphatase"; a survey of the protein and DNA databases revealed 14 members belonging to this group of acid phosphatases (Table VII). The positive charge of the guanido group of arginine is probably responsible for the recognition and anchoring of the negatively charged phosphate group to the proximity of a histidyl residue in the active site. The phosphate group is transiently

TABLE VI

ALIGNMENT OF FUNGAL PHYA AND PHYB ACTIVE-SITE SEQUENCE WITH PUBLISHED ACID PHOSPHATASES, PHOSPHOGLYCERATE MUTASE, AND FRUCTOSE-2,6-BIPHOSPHATASE

AfphyA	(52)	C R V T F A Q V L S R H G A R Y P T D S K G K
AfphyB	(52)	C E V D T V I M V K R H G E R Y P S P S A G K
YscACP3	(46)	C E M K Q L Q M L A R H G E R Y P T Y S K G A
YscACP5	(46)	C E M K Q L Q M L A R H G E R Y P T V S L A K
YscpACP1	(29)	C K I K Q V H T L Q R H G S R N P T G G N A A
ECACP	(6)	L K L E S V V I V S R H G V R A P T K A T Q L
HuPACP	(1)	K E L K F V T L V F R H G D R S P I D T F P T
HuLACP	(1)	R S L R F V T L L Y R H G D R S P V K T Y P K
RatACP	(1)	R S L R F V T L L Y R H G D R S P V K A Y P K
RtF2,6BP	(250)	• • • P R S I Y L C R H G E S E L N L R G R I
YePGM	(1)	• • • • P K L V L V R H G Q S E W N E K N L F
HuPGM-M	(1)	• M A T H R L V M V R H G E T T W N Q E N R F
HuBPGM	(1)	• • S K Y K L I M L R H G E G A W N K E N R F

transferred to the histidine group to form an unstable phosphoenzyme complex before hydrolytic cleavage to form orthophosphate (Van Etten, 1982). This mechanism is reasonable because it is known that a phosphate group attaches to the solitary histidine residue in phosphocarrier protein, HPr of gram negative bacteria (Anderson *et al.*, 1993). In *A. niger* NRRL 3135 phyA inactivation of tryptophan led to catalytic demise (Ullah and Dischinger, 1992). Of the four tryptophans, only Trp25 and Trp267 are in the hydrophilic region; the other two residues are in the hydrophobic region and may not play a role in active site formation.

D. ENZYME ENGINEERING STUDIES

Any future improvement of the kinetic parameters and thermo- and pH stability of phytase will depend on understanding the three-dimensional structure of the biocatalyst. Unfortunately, the tertiary structure of the protein has not been determined. Crystallization of the heavily glysosylated fungal phytase and acid phosphatases is extremely difficult (A.H. J. Ullah, unpublished data). Efforts are now under way to express the cloned gene in bacteria to obtain the unglycosylated form of phytase. If the nonglycosylated isoform of fungal phytase can fold properly in bacteria and exhibit enzymatic activity, then subsequent crystallization and structure elucidation will be meaningful and yield information that could be used to fine tune the three-dimensional structure of the protein. Despite these difficulties, current research in structure refinements is proceeding in three areas: increasing thermostability,

TABLE VII

A SURVEY OF MICROBIAL AND MAMMALIAN ACID OPTIMUM HISTIDINE PHOSPHATASES

Enzyme	Source	EC No.	NCBI Seq. ID	Gene loci	AA	RHG motif	HD motif	Reference
Periplasmic phosphoan hydride phospholydrolase. 6-phytase, pH 2.5 optimum APase	*E. coli*	3.1.3.2, 3.1.3.26	130735		432	Present	Present	Dassa *et al.* (1990)
3-Phytase	*A. niger* NRRL 3135	3.1.3.8	464382	phyA	467	Present	Present	Ullah and Dischinger (1992)
3-Phytase	*A. niger*	3.1.3.8	484414	phyA	467	Present	Present	Van Hartingsveldt *et al.* (1993)
3-Phytase	*A. niger (awamori)*	3.1.3.8	166518	phyA	467	Present	Present	Piddington *et al.* (1993)
3-Phytase, pH 2.5 optimum acid phosphatase	*A. niger* NRRL 3135	3.1.3.2, 3.1.3.8	464385	phyB	479	Present	Present	Ehrlich *et al.* (1993)
3-Phytase	*A. niger (awamori)*	3.1.3.8	464384	phyB	479	Present	Present	Piddington *et al.* (1993)
Acid phosphatase	*Schizosaccharomyces pombe*	3.1.3.2	130719	pho1	453	Present	Present	Elliot *et al.* (1986)
Acid phosphatase	*Saccharomyces cerevisiae*	3.1.3.2	130721	pho3	467	Present	Present	Bajwa *et al.* (1984)

Acid phosphatase	*Schizosaccharomyces pombe*	3.1.3.2	130719	pho1	453	Present	Present	Elliot *et al.* (1986)
Acid phosphatase	*Saccharomyces cerevisiae*	3.1.3.2	130721	pho3	467	Present	Present	Bajwa *et al.* (1984)
Acid phosphatase, thiamine repressible	*Schizosaccharomyces pombe*	3.1.3.2	400839	pho4	463	Present	Present	Yang and Schweingruber (1990)
Human prostatic acid phosphatase	Homo sapien	3.1.3.2	189620		386	Present	Present	Van Etten *et al.* (1991)
Rat prostatic acid phosphatase	Rat	3.1.3.2	206028		381	Present	Absent	Roiko *et al.* (1990)
Mouse lysosomal, acid phosphatase	Mouse	3.1.3.2	52871		421	Present	Present	Geier *et al.* (1991)
Human lysosomal acid phosphatase	Homo sapien	3.1.3.2	130727		423	Present	Present	Pohlmann *et al.* (1988)
Rat lysosomal acid phosphatase	Rat	3.1.3.2	130729		423	Present	Present	Himeno *et al.* (1989)

reducing the molecular mass, and constructing a chimeric enzyme with the acid phosphatase. Enzyme engineering studies with the acid phosphatase, lacking phytate hydrolysis, may include incorporation of the phytase active site sequence in the protein to impart phytate binding and catalysis. The other protein engineering effort may include alteration of the glycosylation signals to understand the role of glycosylation in secretion and stability of the protein. Because data are lacking on the X-ray-deduced three-dimensional structure of any microbial acid optimum phosphomonoesterase, computer-assisted modeling cannot be performed on fungal phytase or acid phosphatase. However, the crystal structure of recombinant rat acid phosphatase containing the active site sequence RHGXRXP had been determined to 3 Å by protein crystallographic methods (Schneider *et al.*, 1993). The protein is built up of two domains: an α/β domain consisting of a seven-stranded β-sheet with helices on both sides of the sheet and a smaller α domain. The topology of one of the domains of acid phosphatase is very similar to the structure of phosphoglycerate mutase (Campbell *et al.*, 1974). The Garnier analysis of phytase also shows similar arrangements of alternating α/β structure (Fig. 6). It is reasonable to predict that the overall structure of fungal phytase will parallel the structure of rat acid phosphatase. Computer-assisted homology modeling of phytase based on the structure of rat acid phosphatase could not be performed at this time (A. H. J. Ullah, unpublished observation).

E. Cloning of Phytase and Acid Phosphatases

1. *USDA Studies*

The group at the Southern Regional Research Center started purification and characterization of the fungal phytase in 1984. The N-terminal and cyanogen bromide fragments of the purified protein were obtained and reported at the poster session of the 9th Enzyme Engineering Conference at Santa Barbara, California in 1987. Researchers from the Gist–brocades were also present at the meeting. Therefore, the sequence information, so vital for the gene cloning, became available to the Gist brocades researchers. The full account of the N-terminal and intersequences was, however, published a year later (Ullah, 1988). The *phyA* gene from *A. niger* NRRL 3135 was partially cloned in a λ gt11 expression library as identified by immunoprobe and sequence verification (Mullaney *et al.*, 1991). The full-length gene was cloned subsequently and the sequence submitted to the GenBank (Accession No. M94550). A second phytase gene (*phyB*) from *A. niger* NRRL 3135 was cloned; this DNA fragment codes for a 479-amino acid enzyme and was found to contain four exons (Ehrlich *et al.*, 1993). An acid phosphatase from *A. niger*

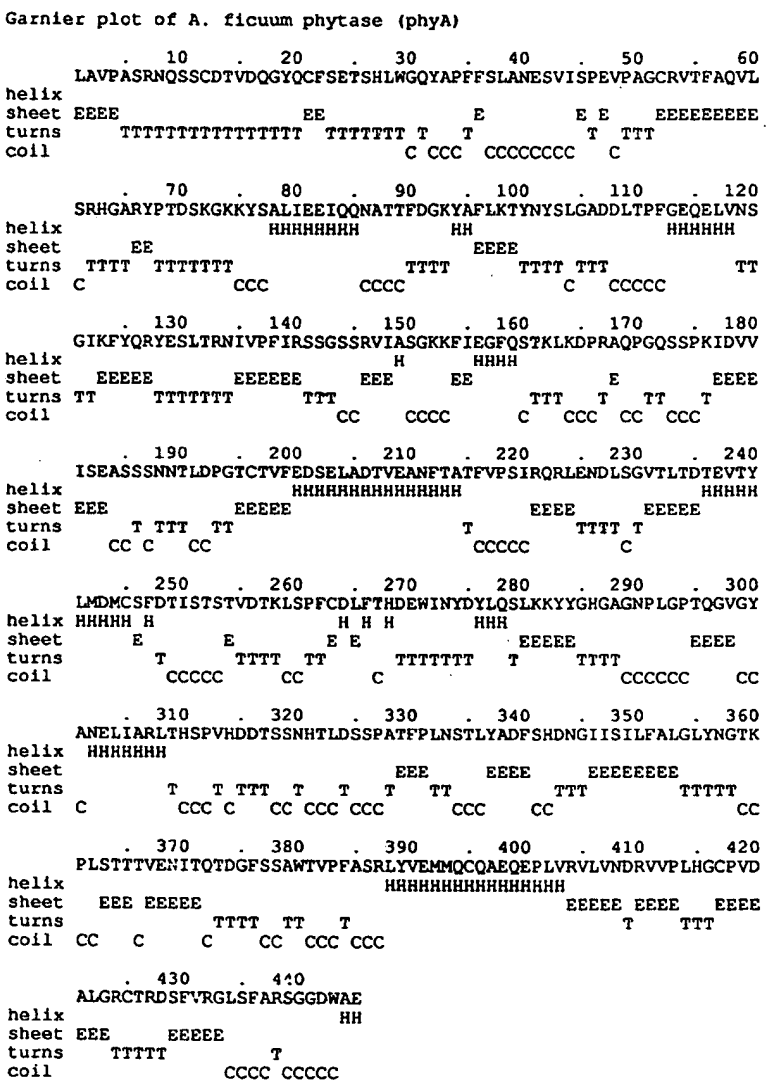

FIG. 6. Secondary structure of phyA as predicted from Garnier plot.

NRRL 3135 that could not be readily separated from pH 2.5 optimum phytase/acid phosphatase (phyB) was cloned and was shown to be homologous to *Penicillium chrysogenum* PHOA (Ehrlich *et al.*, 1994). Very recently, a gene coding for the metalloenzyme pH 6.0 optimum acid

phosphatase was cloned (Mullaney *et al.*, 1995) and shown to be homologous to the aphA gene. The encoded amino acid sequence verified by the chemically deduced sequencing (Ullah *et al.*, 1994).

2. *Dutch Studies*

The Dutch group initiated the cloning work in late 1980s after the biochemical and sequence information was published by the USDA group (Ullah, 1988b). The researchers from the Gist–brocades and TNO Medical Biology cloned and sequenced the gene, and overexpressed the phytase gene (*phyA*) from *A. niger* NRRL 3135. The gene was isolated using degenerate oligodeoxyribonucleotides deduced from phytase amino acid sequences. Nucleotide sequence analysis of the cloned DNA indicated an open reading frame coding for a 467-amino acid protein and included in the DNA is an intron of 102 base pairs in the 5' part of the gene (GenBank Accession No. Z16414). The gene was identical to the one cloned by the USDA group (GenBank Accession No. M94550). However, the major contribution of the Dutch group was the understanding of the expression of the phytase gene at the mRNA level by inorganic orthophosphate levels. The researchers have detected a transcript of 1.8 kb after cell growth in low-phosphate medium. Transcription of *phyA* initiates at least seven start points within a region located 45–25 nt upstream from the start codon. In transformants of *A. niger*, expression of multiple copies of *phyA* gave up to about 10-fold higher phytase activities than the level produced by the native wild-type strain (Van Hartingsveldt *et al.*, 1993).

In other studies, the Dutch group has also expressed the fungal phytase (phyA) in tobacco seeds. The protein was expressed as 1% of the soluble proteins in mature tobacco seeds (Pen *et al.*, 1993). Detailed descriptions of the gene construct used for transformation of tobacco have been published. The molecular weight of the phytase produced in seeds was 67 kDa compared to 80 kDa in *A. niger*. Differences were due to glycosylation. When the cloned plant phyA was deglycosylated and compared to deglycosylated *A. niger* phyA, the molecular weights were identical. The *phyA* gene has been cloned into canola (*Brassica napa*) (Beudeker and Pen, 1994). The transgenic plant seeds of both species have been tested as a source of phytase for monogastric animals and are efficacious.

3. *Pan Labs Studies*

The Pan Labs research with fungal phytase and acid phosphatase stems from the interest of Alko Ltd., Rajamaki, Finland. These groups cloned the genes for phytase and pH 2.5 optimum acid phosphatase from *A. niger* var. *awamori* (Piddington *et al.*, 1993). The genes were isolated from the genomic DNA using oligodeoxyribonucleotide probes based on the protein sequences reported by the USDA group. A potential 102-bp

tial 102-bp intron was identified between the start codon and the N-terminal amino acid residue. Furthermore, a 19-residue signal peptide was identified in the N-terminal segment. The same genomic library was also probed with oligodeoxyribonucleotides based on the pH 2.5 optimum acid phosphatase sequence. One of the clones contained a 2.1-kb fragment that hybridized strongly to two oligonucleotide probes based on different peptides from the same protein. This 2.1-kb fragment contained the sequence for 12 previously sequenced peptides including the N-terminal peptide sequence. The researchers identified intron boundaries through the isolation and sequencing of pH 2.5 optimum acid phosphatase-encoding cDNA. Unlike the phytase gene (*phyA*), three short introns were revealed by the sequencing. The resulting translated sequence codes for a 479-amino acid protein including a 19-amino acid signal peptide. The gene coding for the acid phosphatase (*aph*) is very similar to a gene coding for the second phytase (*phyB*) in *A. niger* NRRL 3135 (Ehrlich *et al.*, 1993).

F. Immobilization Studies with Phytase and Acid Phosphatase

Fungal phytase and acid phosphatases are hydrolytic enzymes with a high catalytic turnover number—typically the range being from 220 to 1000 per second (Ullah, 1988a, Ullah and Cummins, 1987b, 1988b). Thus, these categories of enzymes are the ideal candidates for immobilization and construction of packed-bed bioreactors. Phytases act on myoinositol hexaphosphate to degrade sequentially to liberate orthophosphates, the byproduct being myoinositol penta-, tetra-, tri-, di-, and monophosphate. An efficient bioreactor of the immobilized phytase could be used to produce various species of myoinositol polyphosphates and treat soybean milk to lower the content of myoinositol hexa-, and pentaphosphate. The higher form of myoinositol phosphates, i.e., hexa-, and pentaphosphate, may act as metal chelators, and thus interfere with the mineral nutrition in monogastric animals. The enzymatic action of immobilized phytase on phytate may render the molecule to be a nonchelator by conversion to a lower form of myoinositol tetra-, and triphosphates. *Aspergillus niger* NRRL 3135 phyA was covalently immobilized on Fractogel TSK HW-75 F. A packed-bed bioreactor was constructed with the immobilized enzyme. The catalytic parameters and stability of the immobilized enzymes were determined. No shift in pH optima was observed for the covalently attached phyA compared to the soluble enzyme. The temperature optima shifted from 58° to 65°C. Despite the increase of K^m for phytate and a downward shift in catalytic activity, the immobilized enzyme can hydrolyze over 50% of the orthophosphate from phytate after repeated passage through the bioreactor (Ullah and Cummins, 1987a). When the product of the bioreactor

was characterized by an HPLC on an anion-exchange column, myoinositol penta-, tetra-, tri-, di-, and monophosphates were detected in the eluate. After exhaustive hydrolysis of phytate by the bioreactor, only myoinositol di-, and monophosphates were detected in the eluate (Ullah and Phillippy, 1988). To improve the kinetic parameters of the immobilized biocatalyst, fungal phytase was also covalently attached to glutaraldehyde-activated silicate (Ullah and Cummins, 1988a). About 20% of the total phytase was bound to the activated resin, and the catalytic activity of the bound protein was reduced fourfold. This may have resulted from extensive crosslinking of the phytase to the silicate. In an attempt to immobilize phytase through its carbohydrate moieties rather than protein backbone, the biocatalyst was immobilized on crosslinked agarose (Dischinger and Ullah, 1992). When immobilization was achieved through the protein backbone, myoinositol di- and monophosphates were the predominant species generated on substrate passage. By contrast, when immobilization was performed using carbohydrate side chain, a maximum of 31% of the available phosphate was cleaved. This may be a consequence of a difference in conformation of the catalytic active center, which resulted from differential crosslinking of the enzyme through its carbohydrate moieties. The bioreactor's output and the K_{cat} indicate a diminished catalytic performance. Similar diminished activity was also observed with the immobilized phytase when the attachment was made through the protein backbone. The results of the immobilization study indicate that the active catalytic center of the enzyme may be distorted by extensive crosslinking of proteins to the matrix. Because the native phytase is heavily glycosylated, prevention of extensive crosslinking is difficult. By using site-directed mutagenesis, it should be possible to remove most glycosylation to produce an enzyme that can be immobilized by a few carbohydrate chains while retaining a high level of activity.

VI. Feed Studies with Phytase

A. Early Studies with Poultry

Studies on enzymatic treatment of feed using microbial phytase sources have demonstrated that this method increases the bioavailability of proteins and essential minerals and provides levels of growth performance as good as or better than those with phosphate supplementation. Nelson *et al.* (1968a) were the first to pretreat a corn–soya diet with culture filtrate containing phytase *A. niger* NRRL 3135 to a corn–soya diet and fed it to 1 day old chicks. The chicks showed increases in bone ash due to the phytin-P released from the dietary substances by the enzyme. When the

phytin-P of either soybean meal or cottonseed meal was hydrolyzed by the phytase in the culture filtrate, it was necessary to increase the moisture level to distribute the enzyme within the meal (T. R. Shieh, personal communication). The cost of heat energy to dry the treated meal to prevent subsequent spoilage in a proposed process was higher than the value of phosphorus released. This approach was abandoned.

Nelson *et al.* (1971) added graded levels of solvent-precipitated *A. niger* NRRL 3135 phytase to an experimental and to a commercial corn soybean meal diet and fed it to chicks. They measured bone ash and feed-to-gain ratios. They reported that the enzyme was active in the animals and that they were able to incorporate the released phytin-P into bone. The feed-to-gain ratios were higher than would be expected. They concluded that chicks could utilize the P from phytin-P as well as supplemental P. They also titered the enzyme in the chick and presented data that indicated the units of phytase that would be required to attain maximum phosphorus release from the diets. The maximum amount of phytin-P (2.1–3.0 g / kg of diet) was released if 1500–2000 μm P/hr/ml of phytase was added per kilogram of diet. Nelson *et al.* (1968c) determined the effect of feeding phytase on calcium requirements. They concluded that if 90% of the phytin-P was hydrolyzed by phytase, the amount of calcium from natural ingredients required in the diet was at least one-third less. The superior activity of *A. niger* NRRL 3135 phytase and its practical application to animal feed for the removal of phytic acid has been demonstrated in many experiments (Han, 1989; Han and Wilfred, 1988; Howson and Davis, 1983; Nelson *et al.*, 1971, 1968a; Rojas and Scott, 1968).

B. Recent Studies on Phytase as a Feed Additive

Simons *et al.* (1990), Jongbloed and Kemme (1990), and Jongbloed *et al.* (1992), in The Netherlands, collaborated to reaffirm the pioneering studies of Nelson *et al.* (1968a,b, 1971) of phytase fed to broilers. They not only confirmed the earlier work with the feeding of solvent precipitated enzyme to chick diets, but they also extended the data and were the first to demonstrate the efficacy of feeding phytase to swine. They concluded that the addition of enzyme (1000 μm P/hr/ml of phytase/kg of diet) was sufficient to provide levels of performance equal to or better than that attained by adding supplemental inorganic phosphate to a broiler diet. If the amount of enzyme was increased to 1500 μm P/hr/ml of phytase/kg of diet, the results indicated improved performance compared with birds fed control diets. It is interesting to compare these feed studies with those of Nelson *et al.* (1971). The units of enzyme required for releases of phytin-P in commercial type diets is in

fairly close agreement. They extended their studies and measured the amount of phosphorus in the feces of the broilers fed enzyme. When microbial phytase was fed to low-P diets for broilers, the availability of P increased to 60% and the amount of P in the droppings decreased by 50%. They established the potential benefits that the use of phytase in commercial diets would have on abating phosphate pollution in soil and water pollution.

Royal Gist–brocades, Delft, The Netherlands has conducted and/or has furnished phytase (Natuphos produced by cloning phyA from *A. niger* NRRL 3135 into *A. niger* CBS 513.88) to various researchers for poultry feed studies. The results (Farrell *et al.*, 1993; Von Schoner *et al.*, 1992, Schoner, 1992, Broz *et al.*, 1994) have reaffirmed the earlier studies that the phytin-P in feed ingredients is utilized by monogastric animals fed phytase. They have also extended the studies to layers (Van der Klis and Veersteegh, 1991), ducks and ducklings (Farrel *et al.*, 1993), and swine (Beers and Jongbloed, 1992; von Pallauf *et al.*, 1992; Ketaren *et al.*, 1993; Hoppe *et al.*, 1993; Mroz *et al.*, 1994).

Alko Biotechnology has supplied phytase, Finase F, produced by their cloned version of *A. niger* NRRL 3135 to various researchers who tested its effect in swine (Young *et al.*, 1993; Lei *et al.*, 1993a, 1994; Cromwell *et al.*, 1993). The results utilizing their preparation are consistent with the results using phytase derived from other sources. Lei *et al.* (1993b) demonstrated that the addition of phytase to the diets of weanling pigs improved the bioavailability of zinc and phytate phosphorus.

In some of the studies, data are presented that allow the relationship between units of phytase and P released to be calculated. It is noteworthy that the units of enzyme required for hydrolysis of a set amount of phytin-P are fairly consistent. One can estimate within a fairly narrow range the amount of phytase required to hydrolyze the phytin-P present in almost any diet that is used commercially to rear animals. Depending on the specific diet used in the study, 380–1000 μm P/hr (6308–16600 nKat) of phytase is required to replace 1 g of P supplied from an inorganic source. This value holds from the earliest research using phytase from the original strain to the cloned strains. It is true in broilers, layers, swine, etc. Because some types of diets produce beneficial effects, such as calcium and iron availability, it is necessary to empirically test the absolute levels required if least-cost formulations are to be achieved.

Additionally, the new studies have measured the concentration of phosphorus in the intestinal tract and feces. As such, these studies are very useful in quantifying the effect of feeding the enzyme to poultry and swine and the effect it has on pollution abatement. If a sufficient quantity of enzyme is fed, the calculated P agrees with the decrease in P excreted.

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VII. Economics and Potential Effect of Phytase on Pollution Abatement

One of the driving forces in the development of phytase as a product is the awareness of the environmental effects of the release of concentrated pollutants, especially in intensive agricultural production of animals. In various European countries, especially in The Netherlands and Germany, government has mandated that the amount of manure that may be landspread is governed by the actual needs of the soil and the crop and not by some arbitrary stocking rate (Dunn, 1994). If a livestock producer wishes to increase the size of its animal production unit without increasing the acreage of land on which the manure is spread, it is necessary to decrease the total phosphate and other nutrients in the manure. This necessitates either a more efficient method to utilize the phosphorus supplied to the animal or the necessity to remove the phosphorus from the excrement before discharge to the environment. These mandates in the late 1980s caused a resurgence in the interest in phytase. The economics of the situation was changed. The value of phytase was no longer restricted to the value of phytin-P released or some nebulous benefit from the enzyme preparations not yet identified. A flurry of research activity occurred

If phytase was used as a feed ingredient in the diets of all of the monogastric animals reared in the United States, it would release phosphorus that has a value of $\$1.68 \times 10^8$ (Table VIII). These data are based on

TABLE VIII

ESTIMATE OF PHYTASE MARKET VALUE IN ANIMAL FEEDS

Animal	No. in United States in 1992[a]	Average live wt. (kg)	kg of feed/ animal	Phytin-P conc. in feed (%)	kg Phytin utilized per animal	kg Phytin-P hydrolyzed/ year	Total value of phytin-P ($)
Broilers	6.14×10^9	2.01	3.8	0.21	0.008	4.91×10^7	6.75×10^7
Layers	3.64×10^8		36.4/year	0.18	0.066	2.40×10^7	3.32×10^7
Ducks	1.80×10^7	2.95	7.08	0.21	0.015	2.70×10^5	3.70×10^5
Turkeys	2.89×10^8	9.91	26.4	0.21	0.055	1.59×10^7	2.19×10^7
Pigs	5.78×10^7	80.4	265	0.21	0.56	3.24×10^7	4.44×10^7
Total						1.22×10^{8b}	1.68×10^8

[a] National Agricultural Statistics 1993.

[b] 134,082 Tons.

the most recent livestock production statistics available from the USDA, the current value of inorganic phosphate, the amount of feed required to produce the animals, etc.

The animal feed studies discussed previously have provided the quantitative data on the amount of supplied dietary phosphate assimilated by animals and the diminished amount of phosphorus excreted in the manure. Hoppe *et al.*, (1993); Hoppe (1992); Von Schoner and Hoppe (1992); and Von Schoner *et al.*, (1992) have summarized and conservatively estimated the amount of phosphorus that is assimilated and not excreted when phytase is used in the diets of poultry and swine [e.g., the feeding of phytase to swine caused an average reduction of P excreted in all trials of 56 ± 9% (Hoppe, 1992)]. When those data (Hoppe *et al.*, 1993; Hoppe, 1992; Von Schoner and Hoppe, 1992; Von Schoner *et al.*, 1992) are used as a basis of estimating the reduced phosphate levels excreted in manure and are multiplied by the number of monogastric animals reared in the United States, 8.23×10^7 kg of P would be precluded from entering the environment (Table IX).

TABLE IX

EFFECT OF USE OF PHYTASE ON ABATEMENT OF PHOSPHATE POLLUTION

Animal	No. in United States in 1992[a]	Average live wt. (kg)	kg of feed/ animal	g P excreted/ animal if supplemented with P	g P excreted/ animal if supplemented with phytase	kg P/ year not excreted
Broilers	6.14×10^9	2.01	3.8	14.5	8.4	3.75×10^7
Layers	3.64×10^8		36.4/ year	139/ year	80.5/ year	2.20×10^7
Ducks	1.8×10^7	2.95	7.08	27 (Estimate)	15.6 (Estimate)	2.81×10^5
Turkeys	2.89×10^8	9.91	26.4	101 (Estimate)	58.5 (Estimate)	1.69×10^7
Pigs	5.78×10^7	80.4	265	271 (Estimate)	177	5.62×10^6
Total						8.23×10^{7b}

[a] National Agricultural Statistics 1993.

[b] 90,000 Tons.

Note. 134,082 of P=total value of $\$1.68 \times 10^8$.

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E POLLUTION

g P reted/ mal if pple- ented phytase	kg P/ year not excreted
3.4	3.75×10^7
0.5/ ear	2.20×10^7
5.6 imate)	2.81×10^6
8.5 mate)	1.69×10^7
77	5.62×10^6
	8.23×10^{7b}

VIII. Future Studies

Future studies will probably be concentrated on (i) enzyme engineering to improve the heat stability of the enzyme, reduce the molecular mass, and construct a chimeric enzyme with the acid phosphatase; (ii) elucidation of the 3-dimensional structure of the enzyme and precise glycosylation of the enzyme especially in different plant and microbial systems; (iii) increasing yields in microbial and plant systems by use of various promoters and leader sequences; (iv) application research to find additional uses of the enzyme; (v) basic research on inositol intermediates in plant and animal systems that may create demand for an immobilized enzyme to produce those intermediates; (vi) additional titration of the enzyme in animals for use of least-cost formulations; and (vii) research on delivery systems for the enzyme for use in animal feeds (cloning of the enzyme into various plants high in phytin-P that are used in commercial diets).

IX. Summary

Of all the sources of phytase that have been studied (plant, animal, and microorganisms), the highest yields are produced by a wild-type strain A. *niger* NRRL 3135 (12.7 mg P/hr/ml = 6.8 μm P/ml/min = 113.9 nKat/ml) in a mineral salt medium in which total phosphate (4 mg %) is limiting for growth and cornstarch and glucose are the carbon sources. Synthesis of the enzyme is repressed by phosphate in the wild-type strain.

Aspergillus niger NRRL 3135 produces two phytases one with pH optima at 2.5 and 5.5 (phyA) and one with an optimum at pH 2.0 (phyB). It also produces a pH 6.0 optimum phosphatase that has no phytase activity. These three glycoproteins have been purified to homogeneity, characterized, sequenced, and cloned. The sequences have been compared to each other, other phytases, and to known phosphatases. Their homology has been determined. The active sites of phytases show remarkable homology to the active site residues of the members of a particular class of acid phosphatase (histidine phosphatase). The most conserved sequence is RHGXRXP.

Phytase has been covalently immobilized on Fractogel TSK HW-75 F and glutaraldehyde-activated silicate. It has been immobilized on agarose. Losses of activity have been noted on immobilization but these may be minimized by future research. It should be possible to commercially produce and recover penta-, tetra-, tri-, di-, and monoinositol phosphates using immobilized phytase if markets develop for those products.

Phytase (phyA) from *A. niger* NRRL 3135 has been cloned into an *A. niger* glucoamylase producing strain CBS 513.88 using a construct that has a glucoamylae promoter and an *A. niger* NRRL 3135 leader sequence, and that is devoid of phosphate repression. The yield of the secreted enzyme was increased 52-fold above that of wild-type *A. niger* NRRL 3135. The bioengineered organism produces 270 μm P/ml/min (4500 nKat/ml) which is approximately 7.9 g/liter in the medium. The yield of the secreted enzyme was increased 1440-fold above that of wild type CBS 513.88. Commercial preparations of the cloned enzyme are available.

Phytase (phyA) has been cloned into tobacco and canola. The enzyme is localized in the seed and expressed at high levels. Feeding of the seed to animals has made the phytin-P in the commercial diets available to the animals.

The efficacy of feeding phytase to monogastric animals (poultry and swine) has been established. The amount of enzyme that is necessary to be added to commercial diets has been titred for broilers, layers, turkeys, ducks, and swine. The units of enzyme required are related to the phytin-P content in the diet. The use of the enzyme as a feed additive has been cleared in 22 countries. If phytase were used in the diets of all of the monogastric animals reared in the U. S. , it would release phosphorus that has a value of $\$1.68 \times 10^8$ per year. The FDA has approved the enzyme preparation as GRAS.

The effect of feeding phytase to animals enables assimilation of the P found in feed ingredients and diminishes the amount of phosphate in the manure and subsequently entering the environment. The effect of feeding phytase to animals on pollution has been quantitatively determined. If phytase were used in the diets of all of the monogastric animals reared in the United States, it would preclude 8.23×10^7 kg P from entering the environment.

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Molecular Characterization and Expression of a Phytase Gene from the Thermophilic Fungus *Thermomyces lanuginosus*

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The *phyA* gene encoding an extracellular phytase from the thermophilic fungus *Thermomyces lanuginosus* was cloned and heterologously expressed, and the recombinant gene product was biochemically characterized. The *phyA* gene encodes a primary translation product (PhyA) of 475 amino acids (aa) which includes a putative signal peptide (23 aa) and propeptide (10 aa). The deduced amino acid sequence of PhyA has limited sequence identity (ca. 47%) with *Aspergillus niger* phytase. The *phyA* gene was inserted into an expression vector under transcriptional control of the *Fusarium oxysporum* trypsin gene promoter and used to transform a *Fusarium venenatum* recipient strain. The secreted recombinant phytase protein was enzymatically active between pHs 3 and 7.5, with a specific activity of 110 μmol of inorganic phosphate released per min per mg of protein at pH 6 and 37°C. The *Thermomyces* phytase retained activity at assay temperatures up to 75°C and demonstrated superior catalytic efficiency to any known fungal phytase at 65°C (the temperature optimum). Comparison of this new *Thermomyces* catalyst with the well-known *Aspergillus niger* phytase reveals other favorable properties for the enzyme derived from the thermophilic gene donor, including catalytic activity over an expanded pH range.

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8) catalyze the hydrolysis of phytic acid (*myo*-inositol hexakisphosphate) to the mono-, di-, tri-, tetra-, and pentaphosphates of *myo*-inositol and inorganic phosphate. A broad range of microorganisms, including bacteria (20), yeasts (2), and filamentous fungi (10, 19, 27), produce phytases.

Phytic acid is the primary storage form of phosphate in cereal grains, legumes, and oilseeds, such as soy, which are the principal components of animal feeds. However, monogastric animals are unable to metabolize phytic acid and largely excrete it in their manure. Therefore, the presence of phytic acid in animal feeds for chickens and pigs is undesirable, because the phosphate moieties of phytic acid chelate essential minerals and possibly proteins, rendering the nutrients unavailable. Since phosphorus is an essential element for the growth of all organisms, livestock feed must be supplemented with inorganic phosphate. There are a number of published reports (12, 16, 18, 26) describing the use of phytases in the feeds of monogastric animals and in human food.

When phytic acid is not metabolized by monogastric animals the phosphate level in the manure can also create disposal problems. The amount of manure produced worldwide has increased significantly as a result of increased livestock production. Environmental pollution with high-phosphate manure has caused problems in various locations around the world due to the accumulation of phosphate, particularly in bodies of water. Consequently, animal feed distributors in Europe have begun to formulate feed products with supplemental phytase in order to improve feedlot productivity and decrease phosphate waste. Thus, phytases are also useful for reducing the amount of phytate in manure (13, 18). The current commercial feed supplement is a recombinant *Aspergillus niger* (previously *Aspergillus ficuum*) phytase produced in *Aspergillus niger* (27) or *Aspergillus oryzae* (i.e., Phytase Novo [13]).

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There is a definite commercial need for second-generation phytases with improved properties (e.g., higher thermostability and catalytic efficiency) that can be produced in commercially significant quantities. Our objectives were to identify, clone, and characterize a phytase from a thermophilic fungus in anticipation that this enzyme would offer superior biochemical properties.

MATERIALS AND METHODS

DNA extraction and hybridization analysis. Total cellular DNA was extracted from *Thermomyces lanuginosus* CBS 586.94 by the procedure described by Timberlake and Bernard (21). Genomic DNA samples were analyzed by Southern hybridization (6) under conditions of low stringency (i.e., 5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA {pH 7.7}], 25% formamide, 0.3% sodium dodecyl sulfate [SDS]). A phytase-specific probe fragment comprising the *Aspergillus niger phyA* coding region (approximately 1.6 kb) was radiolabeled by nick translation (11) with [α-^{32}P]dCTP (Amersham, Arlington Heights, Ill.) and added to the hybridization buffer at an activity of approximately 10^6 cpm per ml. The hybridization and washing conditions have been described previously (4).

DNA libraries and identification of phytase clones. Genomic DNA libraries were constructed with the bacteriophage cloning vector λZipLox (Life Technologies, Gaithersburg, Md.) with *Escherichia coli* Y1090ZL cells (Life Technologies) as a host for plating and purification of recombinant bacteriophages and *E. coli* DH10Bzip (Life Technologies) for excision of individual pZL1-phytase clones. Total cellular DNA was partially digested with *Tsp*509I and size fractionated on 1% agarose gels. DNA fragments migrating in the range of 3 to 7 kb were excised and eluted from the gel with Prep-a-Gene reagents (Bio-Rad Laboratories, Hercules, Calif.). The eluted DNA fragments were ligated with *Eco*RI-cleaved and dephosphorylated λZipLox vector arms (Life Technologies), and the ligation mixtures were packaged with commercial packaging extracts (Stratagene, La Jolla, Calif.). The packaged DNA libraries were plated and amplified in *E. coli* Y1090ZL cells (Life Technologies). Approximately 30,000 plaques from the library were screened by plaque hybridization with the radiolabeled phytase probe. One positive clone which hybridizes strongly to the probe was picked and purified twice in *E. coli* Y1090ZL cells. The phytase clone was subsequently excised from the λZipLox vector as a pZL1-phytase clone (5) and designated pMWR46.

Molecular analysis of the *T. lanuginosus* phytase gene. Restriction mapping of pMWR46 was performed by standard methods (11). DNA sequencing of the phytase clones was performed with model 373A automated DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.) by the primer-walking technique with dye-terminator chemistry (7). In addition to the *lac* forward and *lac* reverse primers, specific oligonucleotide sequencing primers were synthesized on an

Applied Biosystems model 394 DNA-RNA synthesizer according to the manufacturer's instructions.

Construction of the phytase expression vector pMWR48. The coding region of the *T. lanuginosus phyA* gene was amplified by PCR with the forward primer 5'-ATTTAAATGGCGGGGATAGGTTTGG-3' and the reverse primer 5'-CT TAATTAATCAAAAGCAGCGATCCC-3'. The sense primer incorporated the first in-frame ATG and extends 16 bp downstream. The antisense primer incorporated a region 14 bp upstream of the translational stop codon and extends through the stop codon. To facilitate the cloning of the amplified fragment, the sense and antisense primers contain a *Swa*I and a *Pac*I restriction site, respectively. The amplified product was digested with *Swa*I and *Pac*I and ligated with pDM181 (also digested with *Swa*I and *Pac*I), a plasmid which provides the *Fusarium oxysporum* trypsin gene promoter and terminator and the *bar* resistance cassette (3). The resulting expression vector was designated pMWR48.

Transformation of *Fusarium venenatum* and analysis of transformants. Transformation protocols and methods for purification of *F. venenatum* (28) transformants are described by Royer et al. (15). Mycelia from primary transformants were used to inoculate shake flasks containing 25 ml of M400 Da medium (50 g of maltodextrin, 2 g of $MgSO_4 \cdot 7H_2O$, 2 g of KH_2PO_4, 4 g of citric acid, 8 g of yeast extract, 2 g of urea, and 0.5 ml of trace metal solution per liter [15]) and incubated with shaking at 30°C. One milliliter of culture supernatant was harvested at 4, 5, and 7 days and stored at 4°C. Phytase activity was assayed as described below. Spores from the primary transformants producing the highest phytase activity were generated by inoculating 20 ml of R medium (12.1 g of $NaNO_3$/liter, 50 g of succinic acid/liter, 20 ml of 50× Vogel's salts, 25 mM $NaNO_3$ [pH 6.0] [15]) with mycelia and incubating it at 30°C with shaking for 2 to 3 days. Single spores were isolated by spreading 150 ml of spore culture onto manipulator plates (1X Vogel's salts, 25 ml of $NaNO_3$, 2.5% sucrose, 2% Noble agar) containing 5 mg of Basta [phosphinothricin or 2-amino-4-(hydroxymethylphosphinyl)butanoic acid; Hoechst-Schering, Rodovre, Denmark] per ml and using a micromanipulator to transfer single spores to a clear region of the plate. After 3 days of growth at room temperature, the germinated spores were transferred to individual Vogel plates containing 5 mg of Basta/ml. Shake flasks containing 25 ml of M400Da medium plus 5 mg of Basta/ml were inoculated in duplicate with mycelial plugs from each single-spore isolate and incubated at 30°C. The best single-spore isolate was selected based on assay of the secreted enzymatic activity, where the transformants produced >150-fold more phytase activity than an untransformed control.

Protein purification. The best *F. venenatum* transformant was run in two 2-liter fermentors with a standard protocol (3). The frozen cell-free broth (1,700 ml) was thawed, clarified by centrifugation, and concentrated on a hollow-fiber Amicon filtration unit with an S1Y10 filter to a volume of 350 ml. The sample was adjusted to pH 7, diluted to a conductivity of 2 mS, and chromatographed at room temperature on a 75-ml-bed-volume Q-Sepharose Big Beads column (Pharmacia), which had been equilibrated in 20 mM Tris-Cl, pH 7. The column was developed at 5 ml/min with the equilibration buffer until the effluent A_{280} had decreased to near baseline. The column was then developed at 5 ml/min with a 600-ml gradient of 0 to 0.6 M NaCl in the same buffer. The bound enzyme activity was found to elute in fractions corresponding to ca. 0.2 M NaCl.

The collected activity peak was concentrated by ultrafiltration with a PM-10 membrane to a volume of 25 ml, diluted to a conductivity of 0.9 mS, and chromatographed at 4 ml/min on a MonoQ HR 10/16 column which had been equilibrated in 20 mM MOPS (morpholinepropanesulfonic acid), pH 7. The column was developed with 80 ml of starting buffer and then with a 400-ml gradient of 0 to 0.5 M NaCl in the same buffer. Enzyme activity was detected in fractions by using the *p*-nitrophenyl phosphate measurement described below. The active fractions were also analyzed with a Novex 10 to 27% gradient SDS-polyacrylamide gel, and the fractions were combined if judged by electrophoresis to be substantially purified.

The peak fractions were combined, concentrated with an Amicon PM-10 membrane by ultrafiltration, and exchanged into 20 mM MES (morpholine ethanesulfonic acid), pH 5.5. The sample conductivity was 1.1 mS. One-third of this sample was chromatographed at 1 ml/min on a Mono S HR 5/5 column (Pharmacia) which had been equilibrated in the same buffer. The column was developed with 5 ml of starting buffer and then with a 25-ml linear gradient of 0 to 0.6 M sodium chloride in the same buffer. The active fractions were combined after electrophoretic analysis to eliminate those which contained trace contaminants.

Physicochemical characterization. Isoelectric focusing (IEF) was performed with a Novex pH 3 to 7 IEF gel according to the instructions of the manufacturer. IEF standards from both Pharmacia and Bio-Rad were used to calibrate the gel.

The protein extinction coefficient was determined experimentally by quantitative amino acid analysis with a Hewlett-Packard AminoQuant system. The analysis assumed 49,700 for the protein molecular weight, based on the translated gene sequence for the mature protein.

Amino-terminal sequence analysis was performed on an Applied Biosystems 476A sequencer.

Enzyme assays. Phytase activity was measured by two different methods. During purification, fractions were rapidly evaluated by measuring the rate of *p*-nitrophenyl phosphate hydrolysis at 405 nm with 10 mM substrate in 0.2 M sodium citrate, pH 5.5, at 30°C with a plate reader (Thermomax; Molecular Devices).

Enzyme kinetics studies performed on purified enzyme samples were accomplished by the assay of inorganic phosphate liberated from corn phytic acid (Sigma catalog no. P 8810). Exhaustive phytate hydrolysis was accomplished by incubating 0.5 or 0.1% phytic acid with enzyme (1 U/ml) in 0.2 M sodium citrate, pH 5.5, at 37°C. Aliquots were removed over a period of 10 h and analyzed (see below) for kinetics of phosphorus release. Ten hours was found to be sufficient for the completion of product formation. Standard enzyme kinetics reactions were carried out for 30 min at 37°C in 0.5% (wt/wt) phytic acid. The reaction was quenched by the addition of an equal volume of 15% (wt/wt) trichloroacetic acid. After cooling, 100 μl of the resulting mixture was diluted in 1 ml of water. The sample was incubated at 50°C for 5 min. Color reagent (1 ml) was added, and the 50°C incubation was continued for 15 min. The absorbance of a 200-μl aliquot was measured at 690 nm with a microplate reader. The color reagent was composed of 6 N sulfuric acid–water–2.5% (wt/vol) hepta-ammonium molybdate–10% ascorbate (aqueous) in a ratio of 1:2:1:1 and was prepared fresh daily. Quantitation was based on a standard curve generated with a 10 mM sodium monobasic phosphate standard. One unit is defined as 1 μmol of inorganic phosphate released per min with 0.5% phytic acid in 0.2 M sodium citrate, pH 5.5, at 37°C.

Steady-state kinetics measurements were made by substrate titration. Phytate concentrations were 2.16, 1.08, 0.541, 0.216, 0.108, and 0.0758 mM for K_m determination. Phytate concentrations of 1.08, 0.541, 0.216, and 0.108 mM in the presence or absence of 1 mM sodium monobasic phosphate were used to evaluate product inhibition.

Thermostability measurement. Phytase samples were dissolved at 100 U per ml in 0.2 M sodium citrate, pH 5.5. One hundred-microliter aliquots of each enzyme solution were incubated for 20 min in a water bath at 37, 45, 50, 55, 60, 65, 70, and 75°C. After the heat treatment, the samples were stored at 0°C until activity assays were performed. Each sample was diluted 1:80 in 0.2 M sodium citrate, pH 5.5, containing 0.01% (wt/wt) Tween 20, and the standard activity assay was performed.

pH-activity measurement. To attain a buffering range between pHs 2 and 7, a three-component 125 mM glycine-acetate-citrate buffer was employed. The buffer components were combined at final concentrations of 42 mM per component, and phytic acid was added as a solid to 1% (wt/wt). This mixture was adjusted to pH 7 with concentrated HCl, and a 10-ml aliquot was taken. This process was repeated for every 0.5 pH units through pH 2.

Enzyme stock solutions of 20 U per ml were prepared in 20 mM MES buffer, pH 5.5. Substrate (1% [wt/wt]; 850 μl) in buffer at a given pH was combined with 100 μl of water and 50 μl of enzyme stock solution and incubated for 30 min at 37°C. Subsequently, the enzyme reaction was quenched with 1 ml of 15% trichloroacetic acid and quantitated by the standard method.

Temperature-activity measurement. Enzyme stock solutions of 12.5 U per ml were prepared in 0.2 M sodium citrate buffer, pH 5.5. Two hundred fifty microliters of 1% phytic acid substrate was added to a 1.7-ml Eppendorf tube followed by 240 μl of 0.2 M sodium citrate buffer, pH 5.5. This solution was vortexed and placed in a water bath at the designated temperature. After 20 min of equilibration in the water bath, the mixture was vortexed and 10 μl of phytase solution was added. The sample was vortexed and incubated in the water bath for an additional 30 min, and then the reaction was quenched with 1 ml of 15% trichloroacetic acid and quantitated by the standard method.

Nucleotide sequence accession number. The complete *phyA* gene sequence has been deposited in GENESEQN as accession no. T90070.

RESULTS

Cloning of phytase gene sequences from *T. lanuginosus*. Southern blotting experiments indicated that an *Aspergillus* phytase gene fragment could be used as a probe to identify phytase gene-specific fragments in *T. lanuginosus* genomic DNA (Fig. 1). We screened 30,000 plaques from a genomic library of *T. lanuginosus* DNA constructed in λZipLox for hybridization with the *Aspergillus* phytase gene probe. Several positive clones were picked and excised by an in vivo-excision protocol (5).

Analysis of the *T. lanuginosus phyA* gene. DNA sequencing of one *T. lanuginosus* phytase clone (pMWR46) showed an open reading frame similar to the *A. niger* phytase gene. The positions of introns and exons within the *phyA* gene were assigned based on comparison of the deduced amino acid sequence with the deduced amino acid sequence of the corresponding *A. niger* phytase gene product. On the basis of this analysis, the *T. lanuginosus* phytase gene is comprised of two exons (47 and 1,377 bp), which are separated by a small intron (56 bp). The size and composition of the intron is consistent with those of other fungal genes (9) in that all contain consen-

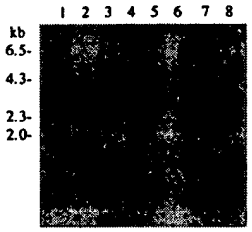

FIG. 1. Autoradiogram from Southern hybridization analysis of *T. lanuginosus* genomic DNA with an *Aspergillus* phytase gene probe. Lanes 1 and 2, *A. niger* genomic DNA digested with *Bam*HI and *Bam*HI plus *Pst*I, respectively; lanes 3 and 4, *Myceliophthora thermophila* genomic DNA digested with *Bam*HI and *Bam*HI plus *Pst*I, respectively; lanes 5 and 6, *Thielavia terrestris* genomic DNA cleaved with *Bam*HI and *Bam*HI plus *Pst*I, respectively; lanes 7 and 8, *T. lanuginosus* genomic DNA cut with *Bam*HI and *Bam*HI plus *Pst*I, respectively.

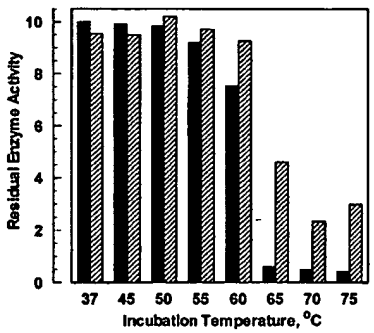

FIG. 3. Phytase thermal stability. Comparison of residual enzyme activity after a 20-min incubation at various temperatures. Full activity corresponds to 10 U. Solid bar, *A. niger* phytase; cross-hatched bar, *T. lanuginosus* phytase.

sus splice donor and acceptor sequences as well as a near approximation of the consensus lariat sequence (RCTRAC) near the 3′ end of each intervening sequence.

The deduced amino acid sequence of the *T. lanuginosus* gene product shows the characteristics of an extracellular fungal enzyme with a cleavable signal sequence. Based on the rules of von Heijne (25), the first 22 amino acids of PhyA likely comprise a secretory signal peptide which directs the nascent polypeptide into the endoplasmic reticulum. Amino-terminal amino acid sequencing suggests that the next 10 amino acids constitute a propeptide which terminates with a dibasic cleavage site (LysLys). The mature PhyA is an acidic protein (predicted isoelectric point, 5.4) composed of 452 amino acids (molecular mass, 51 kDa). The amino acid sequence also contains the active-site motif RHGXRXP, which is shared by other known phytases and acid phosphatases (Fig. 2) (23, 27). Lastly, the deduced amino acid sequence of the mature PhyA has approximately 47.5% identity with the phytase from *A. niger* (GenBank accession no. M94550).

Analysis of *F. venenatum* transformants expressing *T. lanuginosus* phytase. *F. venenatum* has recently been developed as an efficient fungal host for the production of heterologous proteins (15). Culture supernatants from 14 of the 17 primary transformants of pMWR48 were positive when assayed for phytase activity. Two primary transformants with the highest phytase activity were selected for single-spore isolation, and nine single-spore isolates were obtained.

Physicochemical characterization of the recombinant phytase. The purified *T. lanuginosus* phytase was apparently homogeneous in SDS-polyacrylamide gel electrophoresis, with a single component corresponding to a molecular weight of 60,000. The protein sample contained numerous components in IEF analysis ranging from pH 4.7 to 5.2. In contrast to the *T. lanuginosus* phytase, recombinant *A. niger* phytase is composed of a single major component with a pI near 4.9 and two minor bands around pI 4.7.

Amino-terminal sequence analysis of the purified *T. lanuginosus* enzyme identified three components: the major component (ca. 60%) is H_2N-His-Pro-Asn-Val-Asp-Ile-Ala-Arg-His-Trp-Gly-Gln. . ., which corresponds to a Kex2 cleavage site at position 34 in the primary translation product. Two minor sequences, H_2N-Gly-Glu-Asp-Glu-Pro-Phe-Val-Arg-Val-Leu-Val-Asn. . .(ca. 30%) and H_2N-Ser-Glu-Glu-Glu-Glu-Glu-Gly-Glu-Asp-Glu-Pro-Phe. . .(ca. 10%), correspond to internal cleavage sites near the COOH terminal of the protein at positions 428 and 435 in the primary translation product. The observation that our protein sequence data exactly match the predicted translation product of the *T. lanuginosus* gene and the finding that untransformed *Fusarium* host strains produce 2 orders of magnitude less enzyme activity both argue strongly that we have isolated a heterologous gene product.

The specific activities for the two recombinant phytases (i.e., those of *T. lanuginosus* and *A. niger*) were 91 and 180 U/mg, respectively, under standard assay conditions at pH 5.5. At its pH 6 optimum *T. lanuginosus* phytase had a specific activity of 110 μmol of inorganic phosphate released per min per mg of protein at 37°C. Exhaustive enzymatic hydrolysis of phytic acid revealed that *A. niger* and *T. lanuginosus* phytases released identical amounts (70%) of the total theoretically available phosphorus. Steady-state kinetic measurements disclosed that the apparent K_m of *T. lanuginosus* phytase is approximately 110 μM with respect to phytate while *A. niger* has an apparent K_m of 200 μM. There was a faint indication of excess substrate inhibition at the 2.16 mM substrate concentration, perhaps congruent with the report of inhibition above 2 mM for *A. niger* phytase (22). Steady-state kinetics measurements with 1 mM phosphate present failed to reveal any type of inhibition with this product. We estimate that the K_i for phosphate must exceed 3 mM to be undetectable in our experiments. In contrast Ullah (22) has reported that phosphate is a competitive inhibitor, with a K_i of 1.9 mM.

A comparison of enzyme thermostability profiles (Fig. 3) suggests that differences between the stabilities of the two enzymes are small. Neither enzyme is fully inactivated by a high-temperature incubation, and the residual activity profiles are consistent with partially reversible thermal denaturation

Thermomyc PhyA	(33)	C R V E F V Q V L S R H G A R Y P T A H K S E
Myceliophth PhyA	(45)	C E V T F A Q V L S R H G A R A P T L K R A A
Talaromyc PhyA	(54)	C K I T F V Q L L S R H G A R Y P T S S K T E
A.fumigatus PhyA	(44)	C R I T L V Q V L S R H G A R Y P T S S K S K
A.ficuum PhyA	(52)	C R V T F A Q V L S R H G A R Y P T D S K G K
A.ficuum AP2.5	(52)	C E V D T V I M V K R H G E R Y P S P S A G K
YScAP3	(46)	C E M K Q L Q M L A R H G E R Y P T Y S K G A
YScAP5	(46)	C E M K Q L Q M L A R H G E R Y P T V S L A K
HuPAP	(1)	K E L K F V T L V F R H G D R S P I D T F P T
HuLAP	(1)	R S L R F V T L L Y R H G D R S P V K T I P K
E.coliAP	(6)	L K L E S V V I V S R H G V R A P T K A T Q L
CONSENSUS		R H G x R x P

FIG. 2. Alignment of putative active-site regions of acid phosphatases (AP) and phytases from various species. The *M. thermophila* (*Myceliophth*; TREMBL O00107), *Talaromyces thermophilus* (*Talaromyc*; TREMBL O00096), *A. fumigatus* (TREMBL O00092), *A. ficuum* (*A. niger*) (SwissProt P34752 and P34754), *Saccharomyces cerevisiae* (YScAP3 and -5; SwissProt P24031 and P00635), human (HuPAP and HuLAP; SwissProt P15309 and P11117), and *E. coli* (SwissProt P07102) sequences were obtained from the databases indicated. The numbers in parentheses are the starting amino acid positions from the mature proteins for the sequences compared. Identical amino acids are boxed. *Thermocyl*, *T. lanuginosus*.

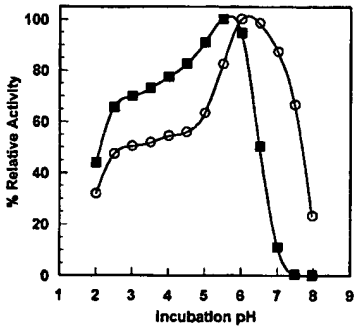

FIG. 4. Phytase pH-activity profile; comparison of relative enzyme activity at various incubation pHs. A relative activity of 100% corresponds to 1 and 1.21 μmol of inorganic phosphate released per min for *A. niger* and *T. lanuginosus* phytases, respectively. Solid square, *A. niger* phytase; open circles, *T. lanuginosus* phytase.

(24). Differential scanning calorimetry (DSC) experiments reveal that the *A. niger* enzyme has a transition at 60°C while *T. lanuginosus* phytase unfolds at 69°C. Others have reported an *Aspergillus fumigatus* phytase which has an apparently greater propensity for reversible thermal denaturation (14), as measured by residual enzyme activity. However, there are no published data on thermal denaturation points for the *A. fumigatus* phytase or other phytase species.

The pH-activity profile comparison of *T. lanuginosus* and *A. niger* phytases indicates substantial similarity between the pH profiles of the two enzymes (Fig. 4). However, the *T. lanuginosus* enzyme is active at neutral pH while the *A. niger* enzyme is not. We could not reproduce the earlier reports (e.g., reference 17) that *A. niger* phytase possesses two pH optima; employing a composite buffer, we measured a broad shoulder near pH 3. We note that there are very few cases of a single enzyme species possessing two pH optima. The earlier reports may originate from impure material which contains traces of the *A. niger* acid phosphatase (29), or they could be artifacts of employing more than one buffer to span the pH range.

Measurement of enzyme activity as a function of temperature revealed a significant difference between the two enzymes (Fig. 5). *T. lanuginosus* phytase has maximum enzyme activity near 65°C and has partial activity even at 75°C. In contrast, *A. niger* phytase is essentially inactive at 65°C. These results are congruent with the DSC data for the two enzymes, which also

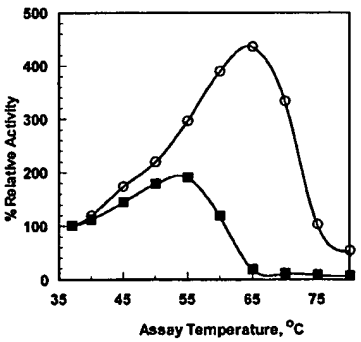

FIG. 5. Phytase temperature-activity measurement; observed enzyme activity as a function of incubation temperature. A relative activity of 100% corresponds to 0.125 μmol of inorganic phosphate released per min. Solid squares, *A. niger* phytase; open circles, *T. lanuginosus* phytase.

indicate a 9°C stability improvement for the *Thermomyces* phytase.

DISCUSSION

Enzyme activity at elevated temperatures may be relevant in applications such as saccharification (a high-temperature industrial process to generate high-fructose corn syrup), where others have reported that the addition of phytase improves carbohydrate yields (1). Figure 5 demonstrates that at 55°C, the optimal temperature for *A. niger* phytase, the *Thermomyces* phytase performs at 79% of the *A. niger* phytase turnover number (despite lower specific activity for *Thermomyces* phytase at 37°C) and at 60°C the *Thermomyces* phytase is operating at 67%-greater catalytic efficiency than the *A. niger* enzyme. The *A. niger* phytase is inactivated at 65°C, where *Thermomyces* phytase activity is maximal.

Enzyme thermal stability is also relevant in animal feed applications, where the enzyme is normally incorporated into the grains prior to pelletization and the feed briefly reaches processing temperatures of 85 to 90°C. In this circumstance a commercial phytase product must be able to withstand brief heating prior to encountering an animal's digestive tract at 37°C. Our physicochemical data demonstrate an improvement of approximately 9°C in denaturation temperature for *Thermomyces* phytase versus the present *A. niger* product.

Animal-feeding trials with formulated phytase supplementation would involve testing a total of 300 broilers or piglets at two enzyme dosages plus a control without enzyme addition. Typically the apparent total-tract digestibility of dissolved matter, organic matter, nitrogen, calcium, and total phosphorus would be monitored at one or two points during an animal's growth to determine the effect of enzyme dosage on feed intake and conversion. Such animal-feeding trials and the level of analysis required to present and evaluate the data are beyond the scope of this paper.

It is tempting to speculate about the structural origins of thermal stability in phytases. However, there is no obvious pattern to the sequence differences between phytases from thermophiles (represented by *Myceliophthora*, *Talaromyces*, and *Thermomyces*) and mesophiles (represented by *A. niger* and *A. fumigatus*). For example, there are no gross differences in protein structure, such as addition or deletion of secondary structure elements. Nor is there a systematic pattern to the sequence differences between the two representative enzymes; i.e., hydrophobic replacements, addition of salt bridges, addition of potential disulfide bonding sites, and deletion of asparagine or aspartate residues are not readily apparent. The most striking difference is the additional consensus N-linked glycosylation site present in the two *Aspergillus* enzymes (sequence position 231 in reference 27) but missing in the three thermophile examples. We believe that the most likely explanation which can be deduced for the sequence differences is derived from evolutionary rather than functional factors.

Recently the discovery of new industrial enzymes has focused on novel microbial sources representing extreme conditions (extremophiles). In many cases the genes encoding these interesting enzymes can be cloned without prior isolation of the catalyst or culturing of the donor microbe. However, heterologous production of the novel enzyme often results in extremely low yields of secreted product or accumulation of inactive material as inclusion bodies. Either of these outcomes is incompatible with the production economics required for commercialization. We have searched for new industrial catalysts from a constellation of thermophilic fungi that are more closely related than the extremophiles to the industrial fungal

production strains which are available. We have successfully isolated enzymes with both improved thermal stability characteristics and the potential for high-level commercial production (4).

T. lanuginosus phytase is an alternative enzyme with performance advantages over the conventional *A. niger* enzyme in the form of stable enzyme activity at elevated temperatures and superior substrate saturation kinetics at physiological pH. A second-generation commercial enzyme may also benefit from protein engineering when a three-dimensional protein structure is available, as is the case for the *A. fumigatus* enzyme (8).

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Isolation, Characterization, Molecular Gene Cloning, and Sequencing of a Novel Phytase from *Bacillus subtilis*

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The *Bacillus subtilis* strain VTT E-68013 was chosen for purification and characterization of its excreted phytase. Purified enzyme had maximal phytase activity at pH 7 and 55°C. Isolated enzyme required calcium for its activity and/or stability and was readily inhibited by EDTA. The enzyme proved to be highly specific since, of the substrates tested, only phytate, ADP, and ATP were hydrolyzed (100, 75, and 50% of the relative activity, respectively). The phytase gene (*phyC*) was cloned from the *B. subtilis* VTT E-68013 genomic library. The deduced amino acid sequence (383 residues) showed no homology to the sequences of other phytases nor to those of any known phosphatases. PhyC did not have the conserved RHGXRXP sequence found in the active site of known phytases, and therefore PhyC appears not to be a member of the phytase subfamily of histidine acid phosphatases but a novel enzyme having phytase activity. Due to its pH profile and optimum, it could be an interesting candidate for feed applications.

Cereals, legumes, and oilseed crops are grown in over 90% of the world's harvested area. These crops serve as a major source of nutrients for humans and animals. An important constituent in these crops is phytic acid (*myo*-inositol hexaphosphate). The salt form, phytate, is the major storage form of phosphorus and accounts for more than 80% of the total phosphorus in cereals and legumes (27). Phytases are enzymes capable of hydrolyzing phytic acid to less-phosphorylated *myo*-inositol derivates. Monogastric animals, such as pig, poultry and fish, are not able to metabolize phytic acid, and therefore inorganic phosphate is added to their diets to satisfy the phosphorus requirement. This consequently contributes to phosphorus pollution problems in areas of intensive livestock production (1, 19, 20). Phytic acid also acts as an antinutritional agent in monogastric animals by chelating various metal ions needed by the animal, such as calcium, copper, and zinc (5, 13, 14). Therefore, the enzymatic hydrolysis of phytic acid into less-phosphorylated *myo*-inositol derivatives in the intestine of monogastric animals is desirable. Many attempts to enzymatically hydrolyze phytic acid have been made to improve the nutritional value of feed and to decrease the amount of phosphorus excreted by animals (12, 24, 32). There have been reports of partially purified microbial phytase preparations from a variety of microbial species (4, 6, 7, 8, 10, 30, 33), the best characterized being those from *Aspergillus ficuum* (34) and *Aspergillus niger* (3). There are two previous reports on partial purification of phytase from *Bacillus subtilis* (26, 31). Genes encoding fungal phytases from *Aspergillus niger* (3, 25, 36), *Aspergillus fumigatus* (22), *Aspergillus terrus* (16), *Myceliophthora thermophila* (16), *Aspergillus nidulans* (23), and *Talaromyces thermophila* (23) have been cloned and sequenced. The only bacterial phytase cloned so far is the *Escherichia coli* gene *appA*, which encodes periplasmic phosphoanhydride phosphohydrolase (2). However, due to the kinetic parameters, this enzyme should be designated a phytase (8).

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In the present study, we screened several food-grade bacterial strains belonging to the genus *Bacillus* for extracellular phytase production. Phytase from the strain showing the highest phytase production was purified and partially characterized, and the gene was cloned, sequenced, and recombinantly produced. Therefore, we report here the first cloned, sequenced, and recombinantly produced food-grade bacterial phytase.

MATERIALS AND METHODS

Chemicals and bacterial strains. Phytic acid, dodecasodium salt, was purchased from Sigma Chemical Co., St. Louis, Mo. Wheat bran was purchased locally (Melia Ltd., Raisio, Finland). All other chemicals were of the analytical grade commercially available. The following strains were obtained from the culture collection of the Technical Research Centre of Finland (VTT): *Bacillus amyloliquefaciens* VTT E-71014, VTT E-71015, VTT E-80124, and VTT E-90408; *Bacillus coagulans* VTT E-82150; *Bacillus licheniformis* VTT E-80117, VTT E-80118, VTT E-80119, and VTT E-83175; *Bacillus stearothermophilus* VTT E-81128, VTT E-81129, VTT E-84208, and VTT E-88318; and *B. subtilis* VTT E-68012, VTT E-68013, VTT E-70009, VTT E-83176, VTT E-83177, VTT E-83178, VTT E-84207, and VTT E-85178. *E. coli* XL-1 Blue MRF′ and SOLR′ (Stratagene, San Diego, Calif.) were used as a host for DNA manipulations and gene expressions. *E. coli* RV308 expression host was obtained from Kristiina Takkinen, Technical Research Centre of Finland. *A. niger* phytase Natuphos was obtained from Gist-brocades, Delft, The Netherlands.

Screening of *Bacillus* strains for phytase production. Strains were tested for phytase production in Luria broth, in Luria broth supplemented with phytate, and in wheat bran extract medium described by Powar and Jagannathan (26). Samples were withdrawn from the culture media at different time points, cleared by centrifugation, and passed through a PD-10 gel filtration column (Pharmacia Inc., Uppsala, Sweden). These crude enzyme preparations were assayed for phytase activity as initially described by Shimizu (31).

Purification of native phytase. All purification steps were carried out at 0 to 4°C unless otherwise stated. Bacteria grown on wheat bran extract were collected by centrifugation at 7,000 × *g* for 30 min. $CaCl_2$ was added to a final concentration of 1 mM in the collected supernatant. The enzyme was precipitated by adding 3 volumes of cold (−20°C) ethanol with constant stirring. Stirring was continued for 45 min, and the precipitation was continued overnight. The precipitate was collected by centrifugation at 1,800 × *g* for 20 min. The collected precipitate was washed once with cold (−20°C) ethanol and once with cold (−20°C) acetone. Excess acetone was evaporated under nitrogen gas flow. The drying was completed by lyophilization. Dried precipitate was dissolved in 100 mM Tris-HCl (pH 7.5) supplemented with 1 mM $CaCl_2$, and then ammonium sulfate was added slowly with constant stirring to give 65% saturation. The solution was incubated overnight and centrifuged at 9,000 × *g* for 60 min, and the supernatant was collected. Ammonium sulfate was added to the supernatant to give 85% saturation. The solution was incubated overnight. Precipitate was

collected by centrifugation at 9,000 × *g* for 60 min. The pellet was dissolved in 100 mM Tris-HCl (pH 7.5) supplemented with 1 mM $CaCl_2$. Aliquots of enzyme preparation were stored at −20°C. For the enzyme assays in defined buffers, an aliquot of the enzyme preparation was thawed and passed through a PD-10 gel filtration column (Pharmacia) into an appropriate buffer. For the enzyme assays in wheat bran buffer systems, aliquots of enzyme preparation were passed through a PD-10 gel filtration column (Pharmacia) into a 100 mM Tris-HCl (pH 7.5) buffer supplemented with 1 mM $CaCl_2$. The molecular weight was determined by using 8 to 25% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Pharmacia). The isoelectric point was determined with the same system by using PhastGel IEF 3-9 isoelectric focusing gels and the Pharmacia IEF calibration kit as the standard. Other protein samples were separated by SDS–12.5% PAGE as described by Laemmli (11) and stained with Coomassie brilliant blue. For sequencing purposes, final purification of the PhyC protein was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) on a 0.21- by 10-cm (TSK Tosoh Corporation, Tokyo, Japan) TMS-250 (C_1) column by elution with a linear gradient of acetonitrile (3 to 100% in 60 min) in 0.1% trifluoroacetic acid. Chromatography was performed at a flow rate of 200 μl/min, and the protein was detected by UV absorbance at 214 nm. The collected protein fraction was dried in a vacuum centrifuge and dissolved in 40 μl of 6 M guanidine-HCl–2 mM EDTA–0.5 M Tris-Cl (pH 7.5).

Production of *B. subtilis* VTT E-68013 phytase in defined media. *B. subtilis* VTT E-68013 colonies were grown on M9 minimal medium, M9 minimal medium supplemented with 2 mM phytate, and phytase screening medium (2% D-glucose, 0.4% sodium phytate, 0.2% $CaCl_2$, 0.5% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot H_2O$ per liter adjusted to pH 7). The culture broth was clarified by centrifugation, proteins were precipitated by adding 3 volumes of cold ethanol (−20°C), and precipitate was dissolved in 100 mM Tris-HCl (pH 7.5) supplemented with 1 mM $CaCl_2$ and assayed for phytase activity.

Alkylation, enzymatic digestion, and peptide separation. Dithiothreitol (5 μmol) was added, and reduction was performed for 20 min at room temperature; this was followed by addition of 1 μl of 4-vinylpyridine (Sigma). Alkylation was performed at room temperature for 15 min, followed by addition of 5 μl of dithiothreitol (1 μmol/μl). The alkylated protein (about 15 μg) was separated from the remaining reagents by C_1 RP-HPLC as described above, dried in a vacuum centrifuge, and dissolved in 50 μl of 0.1 M Tris-Cl (pH 9.2); addition of 0.2 μg of Lysylendopeptidase C (LysC; Wako GmbH, Neuss, Germany) followed. Digestion was performed overnight at 37°C. Generated peptides were separated by narrow-bore RP-HPLC on a 1.0- by 15-cm Vydac C_8 column (300Å, 5 μm; LC-Packings, Amsterdam, The Netherlands). Elution was performed with a linear gradient of acetonitrile (0 to 40% in 120 min) in 0.1% trifluoroacetic acid. Peptides were monitored at 214 nm and automatically collected with a SMART system (Pharmacia Biotech, Uppsala, Sweden).

Mass spectrometry, protein N-terminal sequencing, and internal peptide sequencing. The collected peptides were subjected to MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) mass spectrometry in the delayed extraction mode with a BIFLEX mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) by using a 337-nm nitrogen laser. A thin-layer matrix preparation with saturated α-cyano-4-hydroxycinnamic acid in acetone was used. One-half microliter of matrix was deposited on a stainless-steel target plate and allowed to dry, after which 0.5 μl of sample was added on top of the matrix spot. External calibration was performed with insulin (human; Sigma) and cytochrome *c* (horse heart; Sigma). Protein N-terminal sequencing and internal peptide sequencing were performed with a Procise 494A sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, Calif.).

Phytase activity assays. Enzyme assays were preformed as described by Shimizu (31). One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of P_i per min under assay conditions. The specific activity was expressed in units of enzyme activity per milligram of protein. Enzyme activity assays were performed in defined buffers and in a wheat bran buffer system as described below. All enzyme assays were run in duplicate. Defined buffers used in enzyme activity assays were as follows: 100 mM glycine (pH 3.0), 100 mM succinate (pH 5.0), 100 mM Tris-maleate (pH 5.0, 6.0, 7.0, and 8.0), 100 mM Tris-HCl (pH 7.5, 8.0, and 9.0). All buffers were supplemented with 2 mM sodium phytate and 1 mM $CaCl_2$. Enzyme assays were performed in these buffers at five different temperatures (37, 45, 55, 65, and 75°C). Six-hundred-microliter aliquots of buffer were preincubated at the relevant temperature for 5 min, and the enzyme reactions were started by adding 150 μl of enzyme preparation. Since enzyme addition tends to affect the pH of the reaction mixture, the true pH of each assay mixture was measured at the beginning and at the end of the 30-min incubation. After 30 min of incubation, reactions were stopped with 750 μl of 5% trichloroacetic acid and the released inorganic orthophosphate was measured as described previously (31). The protein concentration of each enzyme preparation was measured with the Bio-Rad protein assay (Bio-Rad Life Science Group, Hercules, Calif.), and the specific activity of enzyme at the different pH and temperature levels was calculated.

Wheat bran extract used in the enzyme activity assay was prepared by dissolving 50 g of wheat bran in 500 ml of distilled water, followed by autoclaving at 121°C for 60 min. The extract was filtered through a cheesecloth, and the volume was adjusted to 500 ml with distilled water and clarified by centrifugation. The supernatant was adjusted to five different pH levels by HCl or NaOH additions (pH 3.0, 5.5, 7.0, 8.0, or 9.0), diluted 1:10 in distilled water, and supplemented with 2 mM sodium phytate and 1 mM $CaCl_2$. Six hundred microliters of the wheat bran buffer described above was preincubated at the desired reaction temperature (37, 55, and 75°C), and the enzyme reactions were then run as described above.

Substrate specificity. Substrate specificity of the PhyC was determined by using the standard activity assay in 100 mM Tris-HCl (pH 7.5) supplemented with 1 mM $CaCl_2$ and 2 mM tested substrate. Besides phytic acid, β-glycerophosphate, D-glucose 6-phosphate, *p*-nitrophenylphosphate, ATP, ADP, AMP, fructose 1,6-diphosphate, 3-phosphoglyceric acid, bis-(*p*-nitrophenyl)phosphate, and α,β-methyleneadenosine-5′-disphosphate were tested as substrates.

General DNA techniques. All PCRs were performed by using a PTC-255 DNA Engine (MJ Research Inc., Watertown, Mass.) and *Taq* polymerase (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, N.J.). On the basis of N-terminal and internal PhyC peptide sequences, several degenerate PCR primers were designed. PCR was performed with these primers by using *B. subtilis* VTT E-68013 DNA as a template at different annealing temperatures and at different magnesium concentrations. The following PCR protocol was chosen: premelting at 94°C for 4 min, followed by 30 cycles of melting at 92°C for 60 s, annealing at 50°C for 60 s, and extension at 72°C for 120 s in 2.5 mM magnesium. The largest PCR fragment was cloned into pCR 2.1 (Invitrogen, San Diego, Calif.) vector and sequenced. Southern blotting was performed as described by Sambrook et al. (29) by using the largest PCR fragment, labelled with digoxigenin (PCR DIG probe synthesis kit; Boehringer-Mannheim, Mannheim, Germany), as the hybridization probe. The nucleotide sequence of the *phyC* gene was determined with the ABI Prism Dye Terminator Cycle Sequencing kit with an ABI 377 DNA sequencer. Nucleotide and amino acid sequence homology searches were performed on National Center for Biotechnology Information (NCBI) databases by Blast searches.

***B. subtilis* VTT E-68013 genomic library construction.** Partially *Eco*RI-digested genomic *B. subtilis* VTT E-68013 DNA was cloned into Lambda ZapII and packaged into lambda particles by using a Lambda ZapII/EcoRI/CIAP GigaPack Gold III cloning kit (Stratagene) as described in the recommendations provided by the manufacturer. Genomic *B. subtilis* VTT E-68013 library was screened with an EasyToHyb hybridization kit (Boehringer-Mannheim) as described in the recommendations provided by the manufacturer by using the largest PCR fragment, labelled with digoxigenin, as the hybridization probe. Positive lambda clones were cored and excised with ExAssist helper phage (Stratagene) to obtain phagemids. The phagemids obtained were transformed into SOLR′ *E. coli* host cells (Stratagene), and plasmid DNA was purified with the Qiagen (Santa Clara, Calif.) plasmid kit and used in analysis of insert DNA and DNA sequencing.

Construction of clones overexpressing recombinant PhyC-His_6 fusion protein. The *phyC* gene fragment encoding mature enzyme was amplified by PCR with insertion of *Sph*I and *Bgl*II sites at the 5′ and 3′ ends, respectively. Primers used were pBsf (5′ CTCGCATGCTGTCCGATCCTTATCATTTTTACCG 3′) and pBsr (5′ GGCAGATCTTTTTCCGCTTCTGTCGGTCAGTTC 3′). The amplified PCR fragment was purified with the QIAquick DNA purification kit (Qiagen) and cloned into *Sph*I/*Bgl*II-cut pQE-70 expression vector harboring C-terminal His_6 tag (Qiagen) to generate plasmid pBsm. Another forward primer, pBssf (5′ CGTTCAATTGAGGAGGAAGTAAAATGAATC 3′), with insertion of an *Mfe*I site (compatible with *Eco*RI), was designed to amplify the *phyC* gene fragment with its natural signal sequence and ribosomal binding site. The reverse primer used in this amplification was the pBsr primer. The amplified PCR fragment was purified with the QIAquick DNA purification kit (Qiagen) and cloned into *Eco*RI/*Bgl*II-cut pQE-70 expression vector harboring C-terminal His_6 tag to generate plasmid pBss. Primer phytac(+) (5′ CGCGGATCCATGG CCCTGTCCGATCCTTATCATTTTACC 3′), with insertion of *Bam*HI and *Nco*I sites, and primer phytac(−) (5′ GCTAGTCTAGATTTTCCGCTTCTGT CGGTCAG 3′), with insertion of an *Xba*I site, was designed to subclone the mature *phyC* gene fragment into *Bam*HI/*Xba*I-cut pUC19. The amplified PCR fragment was cut with *Xba*I and partially cut with *Nco*I due to the internal *Nco*I site in the *phyC* gene. Partially cut fragments were separated on agarose, and the *Nco*I/*Xba*I *phyC* fragment was cut from the gel and purified with the QIAquick DNA purification kit (Qiagen). Purified *phyC Nco*I/*Xba*I fragment was cloned into an *Nco*I/*Xba*I-cut pKKtac *E. coli* expression vector harboring C-terminal His_6 tag to generate pKKtacBs. The pBsm and pBss plasmids were transformed into XL-1 Blue MRF′ as described by Hanahan (9). Plasmid pKKtacBs was transformed into the $CaCl_2$-competent *E. coli* RV308 expression host.

The pBsm and pBss transformants were grown in LB broth containing 100 μg of ampicillin per ml, induced, and purified as described in QIAexpressionist (Qiagen) with growth and induction times varied and temperature as well as the amount of isopropyl-β-D-thiogalactopyranoside (IPTG) used as an inducer to optimize expression. For pKKtacBs expression in RV308, an overnight culture was diluted 1:50 into fresh LB broth supplemented with 100 μg of ampicillin per ml and grown at 37°C and 200 rpm until the A_{600} was 1.0. IPTG was then added to 1 mM, and the culture was shifted to 30°C since the production level of the recombinant PhyC was found to be higher at 30 than at 37°C. After 1 h of induction, $CaCl_2$ was added to 1 mM to stabilize the enzyme produced. For production analysis, samples were withdrawn at various times after induction, cells were pelleted, and recombinant proteins from culture supernatant were purified and assayed for phytase activity. Purification was performed in the following manner: the sample was applied to a Ni-nitrilotriacetic acid matrix

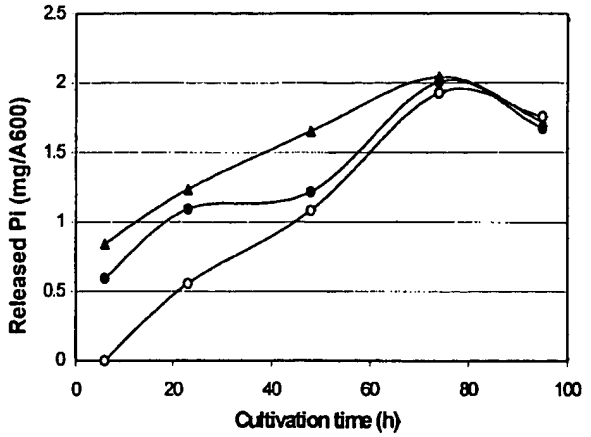

FIG. 1. Phytase activities of *B. subtilis* VTT E-68013 (▲), *B. amyloliquefaciens* VTT E-71015 (●), and *B. amyloliquefaciens* VTT E-90408 (○) during cultivation in wheat bran extract. The phytase activities are expressed as the amount of released inorganic phosphate per cell density (A_{600}). Enzyme assays were run in duplicate, and the standard error in all assays was below 0.04.

(Qiagen) and washed first with 50 mM Tris-HCl–300 mM NaCl (pH 8.0) supplemented with 1 mM $CaCl_2$ and then with 50 mM Tris-HCl–300 mM NaCl (pH 8.0) supplemented with 1 mM $CaCl_2$ and 20 mM imidazole. Recombinant protein was eluted with the same buffer except that the imidazole concentration used was 500 mM.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper has been deposited in the GenBank nucleotide sequence database under accession no. AF029053.

RESULTS

Screening of *Bacillus* strains for phytase production. Twenty-one strains from the genus *Bacillus* were tested for extracellular phytase production in Luria broth, in Luria broth supplemented with phytate, and in wheat bran extract medium. None of the strains produced phytase activity in the Luria broth, whether or not it was supplemented with phytate (data not shown). However, in the wheat bran medium, two *B. amyloliquefaciens* strains and one *B. subtilis* strain produced significant amounts of phytase activity. The amount of inorganic phosphate released per cell density (A_{600}) during the cultivation of these three strains is shown in Fig. 1. The *B. subtilis* strain VTT E-68013 showed the highest phytase activity production and was therefore chosen for phytase enzyme production.

Induction studies. Induction studies were carried out to exclude the possibility that the phytase was readily expressed in phytate-containing media other than wheat bran medium but became instantly and irreversibly inactivated or proteolytically cleaved when secreted to media other than wheat bran. Strain VTT E-68013 was cultivated in a wheat bran extract, Luria broth, Luria broth supplemented with 10 mM phytate, and Luria broth supplemented with 2% bovine serum albumin in order to protect the produced phytase from possible proteolysis. After different time points, samples were withdrawn and assayed for phytase activity. After 50 h of cultivation, when phytase activity was at its highest in wheat bran extract but still no activity was detected in Luria broth, samples of cleared culture media were subjected to SDS-PAGE. At this point of cultivation, cells from each cultivation were examined under the microscope and observed to be undergoing sporulation. No phytase band was detected for any Luria cultivation (Fig. 2), verifying that these media did not support phytase enzyme production even in an inactive form. It was clear that phytate did not induce phytase production but instead proved to repress protein expression of *B. subtilis* VTT E-68013 since the major ca. 58,000 band (most likely amylase) and other bands detected in other cultivation media were barely detectable although cell densities (A_{600}s) in each cultivation were about the same.

To verify that the phytase enzyme was not proteolytically cleaved by excreted proteases in Luria broth, purified phytase was incubated with Luria broth spent medium. There was no drop in phytase activity after 1 h of incubation with Luria broth spent medium at 37°C, indicating that phytase was not cleaved by proteases excreted by *B. subtilis* VTT E-68013.

B. subtilis VTT E-68013 was also grown on defined media. Minimal medium containing inorganic phosphate as well as phytate did not induce phytase production, but defined medium in which phytate was the sole source of phosphate (phytase screening medium; see Materials and Methods) induced phytase production.

Production and purification of native phytase. Phytase proved to be very sensitive to commonly used chromatographic purification methods such as ion exchange and gel filtration. The enzyme required $CaCl_2$ in all purification steps to maintain activity and lost activity if EDTA was used in buffers. A combination of purification by ethanol and ammonium sulfate precipitation proved to be the best purification method and was therefore used to purify protein for enzyme characterization. The purification of phytase is described in Table 1. Redissolved pellet from 85% ammonium sulfate precipitate with high phytase protein purity, but not optimum specific activity, was used in all enzyme characterization experiments described and, after further purification (described in Materials and Methods), was used in N-terminal and internal peptide sequencing. Phytase purification was monitored by SDS-PAGE as shown in Fig. 3.

Chemical and physical characteristics and substrate specificity of the purified phytase. The molecular mass of the mature PhyC was 43 kDa as determined by SDS-PAGE (Fig. 3). The determined molecular mass was 5 kDa more than that of the phytase purified from *B. subtilis* (natto) N-77 described by Shimizu (31). The isoelectric point of PhyC was 6.5 as deter-

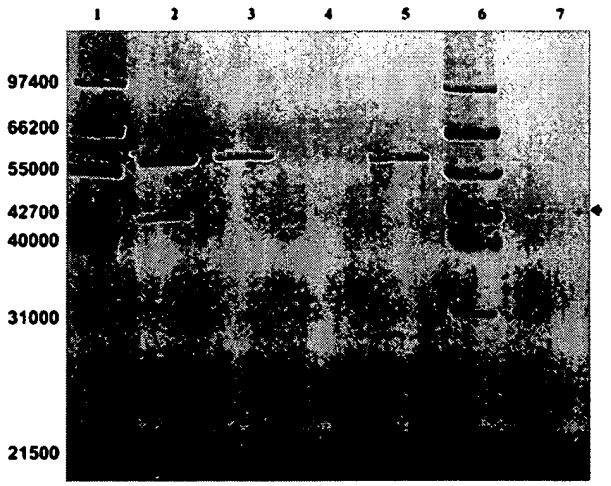

FIG. 2. SDS-PAGE separation of culture supernatant samples of secreted protein of *B. subtilis* VTT E-68013 in different culture media after 50 h of cultivation. Lanes: 1 and 6, molecular weight markers; 2, wheat bran; 3, Luria broth; 4, Luria broth supplemented with 10 mM phytate; 5, Luria broth supplemented with 2% bovine serum albumin; 7, purified recombinant phytase (phytase band indicated by arrow).

TABLE 1. Purification of phytase from *B. subtilis* VTT E-68013

Enzyme sample	Sp act (U/mg)[a]	Recovery (%)	Purification factor (fold)
Culture supernatant	8	100	1
Redissolved ethanol precipitate	15	93	1.9
Supernatant from 65% ammonium sulfate	88	56	11.2
Redissolved pellet from 85% ammonium sulfate	29	22	3.7

[a] One unit is defined as the amount of enzyme required to liberate 1 μmol of P_i per min under assay conditions. The specific activity is expressed in units of enzyme activity per milligram of protein in an activity assay.

mined by isoelectric focusing (data not shown). PhyC proved to be highly specific for phytate, hydrolyzing in addition to phytate only ATP and ADP (50 and 75% of the activity with phytate, respectively) of the substrates tested (see Materials and Methods).

Effect of pH and temperature on the phytase activity. The activity of native PhyC was determined at different pHs and different temperatures as described in Materials and Methods. During the reaction, the changes in pH proved to be insignificant whether the reaction was performed in defined buffer or in wheat bran extract. The final pHs were plotted, and these were within 0.3 pH unit of the initial pH. Figure 4 shows the effect of pH on phytase activity in defined buffers at different temperatures (for clarity, only data for temperatures of 37, 55, and 75°C are shown). The optimum temperature proved to be 55°C. Irrespective of the reaction temperature, PhyC showed the highest phytase activity at neutral pH.

We also determined the PhyC pH activity profiles in a wheat bran buffer system because it is likely to provide an environment somewhat closer to that encountered in feed applications. The optimum pH and temperature as well as the pH profiles as a whole in the wheat bran extract buffer system proved to be very similar to those determined in defined buffers.

To compare PhyC to commercially available fungal phytase used in feed applications, the pH activity profile of Natuphos (an *A. niger* phytase) was also determined. Figure 5 shows the pH activity profiles of PhyC and Natuphos in a wheat bran

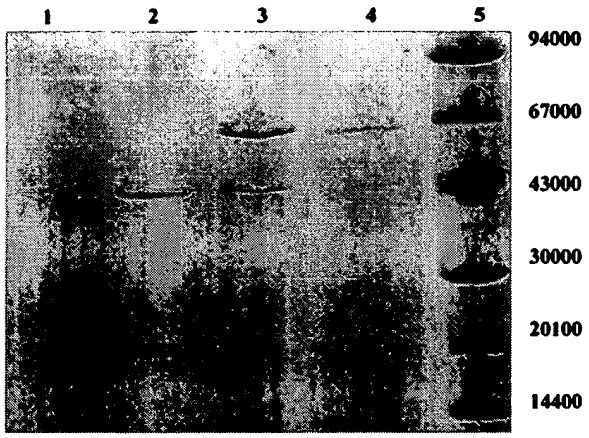

FIG. 3. Purity monitoring of the PhyC by SDS-PAGE. Lanes: 1, 85% saturation ammonium sulfate precipitate; 2, 65% saturation ammonium sulfate supernatant; 3, ethanol precipitate; 4, culture supernatant; 5, molecular weight markers.

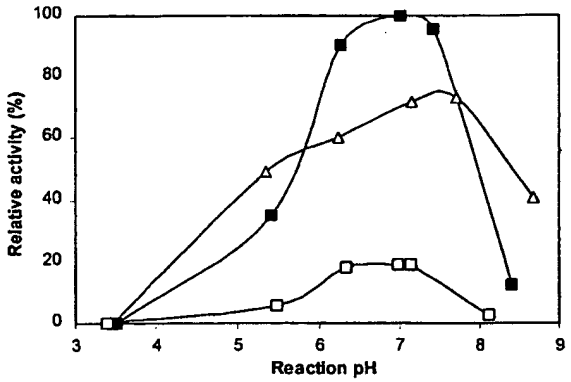

FIG. 4. Effect of pH on phytase activity on defined buffers at three different temperatures (37°C [△], 55°C [■], and 75°C [○]). Phytase activities are expressed as relative activity. Enzyme assays were run in duplicate, and the standard error in all assays was below 0.04.

buffer system at 55°C (optimum temperature of Natuphos as well). Figure 5 clearly shows that PhyC is functional at neutral pH whereas *A. niger* phytase is completely inactive.

N-terminal and internal peptide sequencing and degenerate primer design. The sequence of 25 amino acid residues was obtained from protein N-terminal sequencing. A total of nineteen RP-HPLC-purified internal peptides from alkylated, LysC-digested PhyC was sequenced. The molecular weights of the peptides were measured with a mass spectrometer and compared with calculated molecular weights. LysC digestion was also performed on nonalkylated PhyC; this was followed by RP-HPLC purification of peptides. There was no difference between RP-HPLC results for alkylated and nonalkylated LysC-digested PhyC, indicating the absence of sulfur bridges. Fourteen sequenced internal peptides including the N-terminal peptide showed no overlap with one another and gave a total of 227 amino acid residues. On the basis of these peptide sequences, degenerate primers for PCR were designed. All sequenced peptides and the degenerate primers designed are shown in Table 2.

Molecular cloning and nucleotide sequence of the gene encoding PhyC. PCR was performed with designed degenerate primers by using genomic *B. subtilis* VTT E-68013 DNA as the template. Under PCR conditions described in Materials and

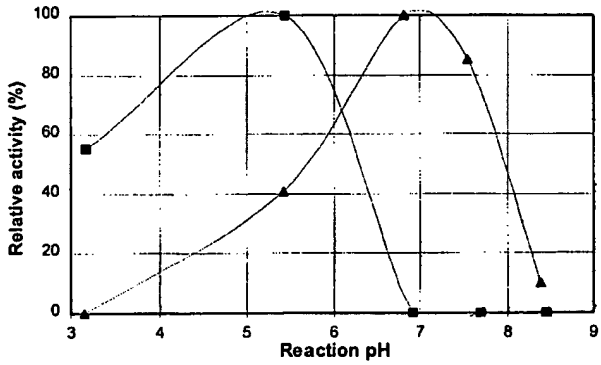

FIG. 5. The pH activity profiles of Natuphos (■), an *A. niger* phytase, and PhyC (▲) at 55°C in a wheat bran buffer system. Phytase activities are expressed as relative activities. Enzyme assays were run in duplicate, and the standard error in all assays was below 0.04.

TABLE 2. N-terminal and internal peptide sequences of PhyC and degenerate primers for PCR designed thereof

Mol wt of peptide		Amino acid sequence[b]	Degenerate primer	
Determined[a]	Calculated		Nucleotide sequence[c]	Designation
		LSDPYHFTVNAAAETEPVDTAGDAA	TCIGATCCITATCATTTTACIGT	6465
		LSDPYHFTVNAAAETEPVDTAGDAADDPAILD		
932	932.1	YYAMVTGK	TTTICCIGTIACCATIGC	6544
1,271.4	1,271.3	EGEFEQYELK	TTCATA(T/C)TGTTCAAATTCICC	6472
1,050.3	1,050.2	MLHSYNTGK	TTICCIGT(A/G)TTATAIGAATGIA(A/G)CAT	6473
798.9	798.9	IVPWER		
2,951.2	2,948.4	IVPWERIADQIGFRPLANEQVDPRK	TGATCIGC(G/A)ATIC(G/T)TTCCCA	6470
		NGTLQSMTDPDHPIATAINEVYGFTLWHSQ	GC(G/A)AT(C/A)GGATGATC(C/A)GGATC	6471
		YVADFRITDGPETDGTSDDDGII	TCIGATTCIGGICCATCIGT	6468
775.7	775.8	LTDRSGK	TTTICCI(G/C)(T/A)IC(G/T)ATCIGT	6543
1,317.9	1,317.4	VDIAAASNRSEGK	CTTCIGAIC(G/T)(G/A)TTIGAIGCIGC	6469
2,167.4	2,167.4	IADQIGFRPLANEQVDPRK		
720.7	720.8	ANQNFK	TTTAAA(G/A)TT(C/T)TG(G/A)TTIGC	6541
619.6	619.7	VRAFK		
		LNNVDIRYDFP	AG(C/A)GGAAAATCATAIC(C/T)(G/A)ATATC	6467
1,779.4	1,778	LNNVDIRYDFPLNGK		
1,236.3	1,236.4	NTIEIYAIDGK	CCATC(G/A)ATIGCATA(G/A)ATTTC	6474
1,137.4	1,137.3	SGLVVYSLDGK	TTICCATCIA(G/T)I(G/C)(T/A)ATAIAC	6542
		FSAEPDGGSNGTVIDRADGRHL	CCATCIGCIC(G/T)ATC(G/A)ATIAC	6475

[a] Molecular weight of peptide determined by mass spectrometer.
[b] Sequences of LysC-digested purified peptides, the first one being the N-terminal peptide. The region from which the degenerate primer was designed is underlined.
[c] Designed degenerate primers, the first one being the forward primer and the others being reverse primers. I, inosine. All primer sequences are written in 5'→3' direction.

Methods, nine reverse primers amplified a single fragment with the forward primer 6465. Primers 6465 and 6470 amplified the largest PCR fragment, which was cloned to a pCR 2.1 vector and sequenced. This resulted in determination of the partial phytase gene sequence of 989 bp. This partial gene fragment was translated into an amino acid sequence, revealing an open reading frame of 330 amino acid residues, and corresponded to the peptide sequences obtained from N-terminal and internal peptide sequencing of purified PhyC. A total of 14 peptides were found in the translated amino acid sequence. Southern hybridization revealed two fragments of 6 and 2.4 kbp, respectively. The genomic *B. subtilis* VTT E-68013 library was screened, and positive clones carrying 6 and 2.4 kbp inserts were obtained. Sequences from these clones were determined by using both vector-specific and gene-specific primers. The sequence of the *phyC* gene, the deduced amino acid sequence, putative −35 and −10 sequences, a ribosomal binding site, and a transcription terminator are shown in Fig. 6. The −35 sequence was the consensus sequence for *Bacillus* expression. However, the −10 sequence was not similar to anything listed (17), suggesting a specific sigma factor. The stop codon (TAA) is followed by a sequence of dyad symmetry (18-nucleotide perfect repeat) which could form a stem-loop structure and therefore be a transcription terminator. The putative ribosomal binding site is 9 nucleotides in length, contains a canonical GGAGG consensus sequence, and is optimally spaced from the start codon (37).

Deduced amino acid sequence of PhyC. The primary amino acid sequence deduced from the nucleotide sequence of the *phyC* gene revealed a fragment of 383 amino acid residues after putative ribosomal binding. The putative signal peptide cleavage site according to Nielsen et al. (21) is located between residues 26 and 27 (SQA-KH). The N-terminal sequence analysis of the purified protein would indicate that the first 29 amino acids are cleaved, but it is possible that the first 26 amino acids are a true signal peptide since the first 26 amino acids closely match the consensus of secreted proteins. Residues 27 to 29 might be analogous to a propeptide because of the positively charged residues (KHK). However, three amino acids would be very short for a propeptide (18). The molecular masses of PhyC preprotein and mature PhyC as deduced from the amino acid sequence were ca. 41.9 kDa and ca. 39 kDa (i.e., without the first 29 residues), respectively. The deduced amino acid sequence was compared to the NCBI protein database by Blast search. The only sequence with homology to PhyC was a hypothetical open reading frame (73% amino acid identity and 86% amino acid similarity to PhyC) from sequence analysis of the *B. subtilis* chromosome region between the *odhAB* and *sspC* loci cloned in a yeast artificial chromosome (38). This hypothetical open reading frame is identical to a hypothetical partial open reading frame (orf181) from the *B. subtilis cgeAB* gene cluster region (28).

Overexpression and purification of recombinant PhyC-His$_6$ fusion proteins. The *phyC* gene fragment encoding mature PhyC (clone pBsm) as well as a fragment encoding mature PhyC with its own signal peptide (clone pBss) was cloned into an overexpression vector, pQE-70, as a C-terminal His$_6$ tag fusion protein, under the control of T5 promoter as described in Materials and Methods. The pBsm clone overexpressed a fusion protein which had the same molecular mass as native, mature protein as determined by SDS-PAGE (data not shown). No active form was obtained from this construct under the expression conditions tested. It appeared that the fusion protein encoded by the pBsm construct was toxic to *E. coli*, since the growth rate of the expression strain XL-1 Blue MRF' harboring plasmid pBsm after induction was significantly lower than that of the strain carrying the vector alone. This is possibly due to the ATPase and ADPase activities of PhyC enzyme. Furthermore, more than 90% of the expressed fusion protein was found in the insoluble cytoplasmic fraction resulting from the formation of inclusion bodies. Likewise, no active enzyme was obtained with the pBss construct that was designed to direct the fusion protein to the periplasmic space by using the natural signal peptide of PhyC. A fusion protein encoded by pBss construct having a molecular mass about 3 kDa larger than that of the nonrecombinant protein as determined by

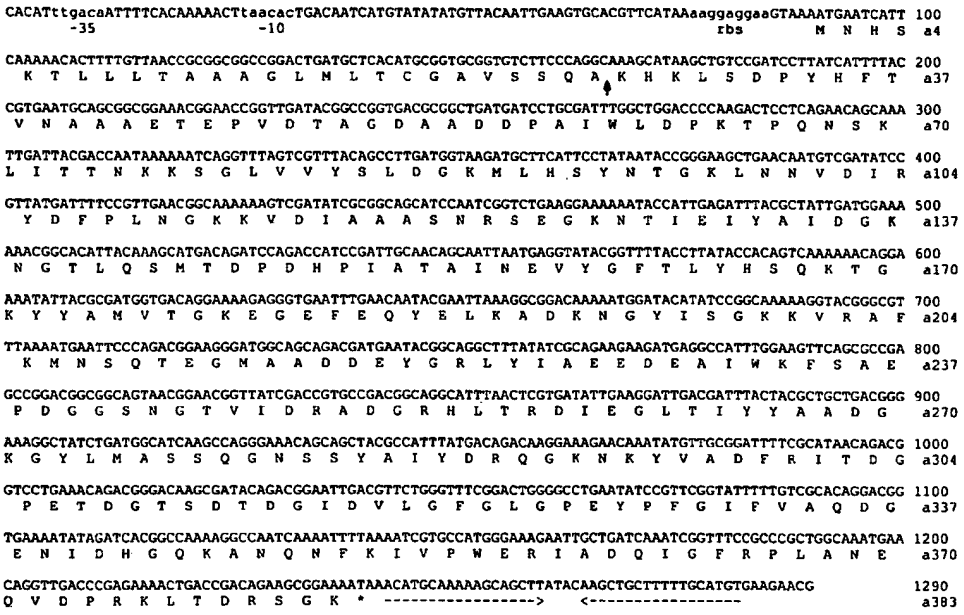

FIG. 6. Nucleotide sequence and deduced amino acid sequence of the *phyC* gene. The putative −35 and −10 sequences are indicated (sequence in lowercase letters) as well as a putative ribosome binding site (rbs; sequence in lowercase letters). A possible transcription terminator downstream of *phyC* gene is indicated by horizontal arrows under the sequence. The possible signal peptide cleavage site is indicated by a vertical arrow.

SDS-PAGE was also found in the insoluble cytoplasmic fraction, indicating that the PhyC signal peptide was not able to direct fusion protein to the periplasmic space of the *E. coli* host strain (data not shown). However, the pKKtacBs construct, in which the *phyC* gene fragment encoding the mature enzyme was cloned downstream of a pectate lyase (*pelB*) signal sequence from *Erwinia carotovora*, overexpressed a fusion protein in an active form to the culture medium of *E. coli* RV308 host. The highest phytase activity was obtained after 20 h of induction at 30°C. The fusion protein was purified from the culture medium as described in Materials and Methods. It had the same molecular mass as that of the native, mature protein as determined by SDS-PAGE (Fig. 2), and it also showed the same pH and temperature optima and the same substrate specificity.

DISCUSSION

The chemical, physical, and enzymatic characteristics of the purified PhyC as well as the requirement for calcium and the inhibition by EDTA suggest that this phytase is similar to the phytase from *B. subtilis* (natto) N-77 described by Shimizu (31) and that from *B. subtilis* described by Powar and Jagannathan (26). It could be assumed that the phytase gene is present in the genome of *B. subtilis* since DNA sequences homologous (67% on a DNA level) to *phyC* from other *B. subtilis* strains have been reported (28, 38). *B. subtilis* VTT E-68013 is not likely very close to *B. subtilis* 168 since another gene cloned from the same genomic *B. subtilis* VTT E-68013 library proved to be only 80% identical to the corresponding gene from *B. subtilis* 168 on a DNA level (15).

The fact that commonly used defined and complex media containing inorganic phosphate in the presence or absence of phytate did not induce the production of PhyC from *B. subtilis* VTT E-68013 suggests that synthesis of PhyC is not upregulated only by phytate itself. However, phytate as a sole source of phosphate induced PhyC production. This finding suggests that PhyC production is induced only when inorganic phosphate is a limiting factor.

All cloned and sequenced microbial phytases have significant homology to each other, and their active sites show remarkable homology to the active site residues of the members of a particular class of acid phosphatases (histidine acid phosphatases), therefore forming the phytase subfamily of histidine acid phosphatases (16, 23, 35). The deduced amino acid sequence of PhyC did not have homology to the sequences of any phytases nor to those of any phosphatases listed in the databases. Most of all, PhyC did not have the RHGXRXP sequence which is the most conserved sequence in the active site of cloned phytases (35); thus, PhyC is not a member of the phytase subfamily of histidine acid phosphatases but is a novel enzyme having a phytase activity.

The inhibition of PhyC by EDTA and the requirement for calcium indicate the presence of a metal, most likely calcium, in the enzyme. We are currently working on identification of this metal, something that is important to know if the PhyC enzyme is to be used in animal feed applications.

ACKNOWLEDGMENTS

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            van Loon,A.P.
  TITLE     Cloning of the phytases from Emericella nidulans and the
            thermophilic fungus Talaromyces thermophilus
  JOURNAL   Biochim. Biophys. Acta 1353 (3), 217-223 (1997)
  MEDLINE   98007872
   PUBMED   9349716
REFERENCE   2  (residues 1 to 466)
  AUTHORS   Pasamontes,L.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-1996) Luis Pasamontes, VFCB, F.Hoffmann-La Roche
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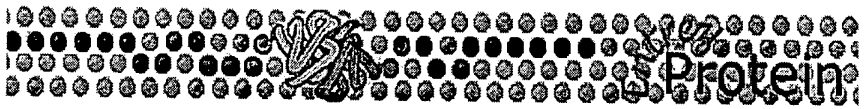

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REFERENCE   1  (sites)
  AUTHORS   Piddington,C.S., Houston,C.S., Paloheimo,M., Cantrell,M.,
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  TITLE     The cloning and sequencing of the genes encoding phytase (phy) and
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  JOURNAL   Gene 133 (1), 55-62 (1993)
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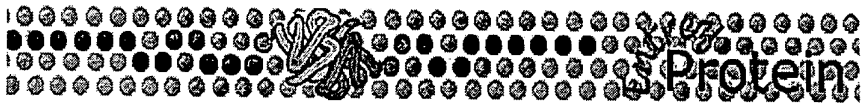


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  ORGANISM  Aspergillus ficuum
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REFERENCE   1  (residues 1 to 441)
  AUTHORS   Ullah,A.H. and Dischinger,H.C. Jr.
  TITLE     Aspergillus ficuum phytase: complete primary structure elucidation
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  JOURNAL   Biochem. Biophys. Res. Commun. 192 (2), 747-753 (1993)
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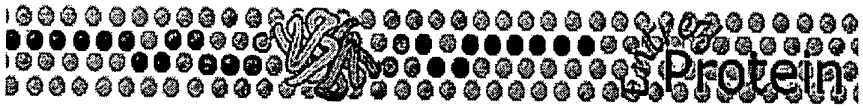


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REFERENCE   1  (residues 1 to 463)
  AUTHORS   Pasamontes,L., Haiker,M., Henriquez-Huecas,M., Mitchell,D.B. and
            van Loon,A.P.
  TITLE     Cloning of the phytases from Emericella nidulans and the
            thermophilic fungus Talaromyces thermophilus
  JOURNAL   Biochim. Biophys. Acta 1353 (3), 217-223 (1997)
  MEDLINE   98007872
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REFERENCE   2  (residues 1 to 463)
  AUTHORS   Pasamontes,L.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-1996) Luis Pasamontes, VFCB, F.Hoffmann-La Roche
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